CHARACTERIZATION OF DEVELOPMENTAL MUTANTS OF PHOMA MEDICAGINIS

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CHARACTERIZATION OF DEVELOPMENTAL MUTANTS OF PHOMA MEDICAGINIS

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Abstract: *Phoma medicaginis* causes spring black stem and leaf spot, an important disease of alfalfa and annual medics. *P. medicaginis* forms uninucleate conidia in melanized pycnidia and is genetically tractable using *Agrobacterium* mediated transformation (ATMT), resulting in random integration of T-DNA that occasionally generates pycnidial mutants. One mutant (P265) displays smaller pycnidia and more aerial hyphae than the wild type. A single T-DNA disrupts a putative poly(A) RNA polymerase gene, Pm*CID13*, which in yeasts interacts with ribonucleotide reductase (RNR). As in yeast mutants, P265 shows sensitivity to hydroxyurea (HU), a RNR inhibitor. To functionally characterize Pm*CID13*, targeted PmΔ*cid13* mutants were created using a hygromycin selectable marker flanked by 1 Kbp regions of Pm*CID13*. PmΔ*cid13* mutants possess similar morphological features to those of P265. The complementation vector pCAM-NAT (nourseothricin selection) was constructed and used to introduce full-length Pm*CID13* into mutants. Complemented mutants showed partial recovery of wild type and often lost the original T-DNA due to homologous integration. To our knowledge, this is the first *CID13* ortholog to be examined in a filamentous fungus. One T-DNA-tagged mutant produced conidia in non-melanized (hyaline) pycnidia. Pycnidial melanization recovered if the mutant was supplemented with phenoloxidase substrates or allowed to age. DNA sequences flanking the insertion did not predict any disrupted open reading frames (ORF) unless a Coccidioides prediction algorithm was used. The Coccidioides predicted ORF (CPO) was expressed in the wild type, but not the mutant (cpo), and has not been annotated in any genomes, to date. Expression of two conserved genes flanking the T-DNA-disrupted cpo was unchanged from the wild type. Knockout of cpo strain displayed same cultural phenotype (non-melinized pycnidia). Complementation of cpo strains with wild type CPO partially recovered pycnidial melanization. Both knockout and complementation transformants were confirmed using RT-PCR. CPO appears to be a novel regulator of pycnidium specific melanization.

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CHAPTER I

INTRODUCTION

Spring black stem and leaf spot, caused by *Phoma medicaginis* Malbr. & Roum., is one of the most significant diseases on *Medicago* spp. in North America, South Africa, Europe, and Australia (Sampson, 1941; Graham, 1979; Barbetti, 1983; Lamprecht, 1984). Spring black stem is characterized by stem and leaf necroses, which can result in almost complete defoliation, thus reducing yield of forage and seed (Johnson, 1933; Richaeds, 1934; Peterson and Melchers, 1942; Rosembilt, 1950; Kerenkamp, 1953). In cases of severe disease, the decay of the crown and upper root of mature plants can occur (Graham, 1979).

Phoma medicaginis is an asexually reproducing dothideomycete and necrotrophic parasite of host plants. It can produce the fungal metabolite brefeldin A to fungitoxic levels. This substance is thought to play a role in defending colonized *Medicago* leaves from competing saprotrophic fungi, as *P. medicaginis* enter the saprotrophic phase of its life-cycle (Weber, 2004).

Medicago species are part of the legume family, Fabaceae. Members of this family include important agricultural taxa that provide oil and protein for human food and livestock feed. Legumes are a major source of soil nitrogen resulting from the symbiotic fixation of

atmospheric nitrogen in specialized root organs called nodules, in which rhizobial bacteria reside (Cook, 1999). *Medicago truncatula* has been studied as a model for the genetic and molecular mechanisms unique to legumes, especially the mutualistic interactions with nitrogen-fixing rhizobia, such as *Sinorhizobium meliloti* (Dangeard) De Lajudie, the rhizobium known to infect *M. truncatula*. The numerous genetic (numerous mutants) and genomic (genome and EST sequences and microarrays) resources available for both *M. truncatula* and *S. meliloti* make this an attractive model system to functionally test the candidate genes controlling important phenotypes of interest such as, rhizobial symbiosis.

Numerous fungal pathosystems of genomic model hosts have been studied, such as, *Cladosporium fulvum* Cooke on tomato (*Lycopersicon esculentum* Miller), *Leptosphaeria maculans* (Desm.) Ces. & De Not. on *Arabidopsis thaliana* (L.) Heynh., and *Magnaporthe grisea* (Hebert) Barr on rice (*Oryza sativa* L.). In order to better understand fungal pathogenicity on legumes, the foliar ascomycetes, *Ascochyta rabiei* (Pass.) Labr. and *Colletotrichum trifolii* Bain have been shown to be genetically manipulable to *Agrobacterium*-mediated transformation. However, such pathosystems have not been well studied in model legumes, such as *M. truncatula* or *Lotus japonicus* (Regel) Larsen (Hiroyuki, 2004; White, 2006). Recently, *Fusarium oxysporum* was proposed as a tractable pathosystem for *M. truncatula* (Ramírez-Suero et al., 2010).

The characterization of molecular interactions between host and pathogen includes identification of pathogen genes encoding virulence factors and the host genes with which they interact. To facilitate studying such mechanisms of pathogenicity, the host and pathogen should both be tractable (model) organisms in order to provide researchers with multiple approaches of investigation. One approach to characterizing disease-related genes is

to identify pathogen mutants with reduced or no pathogenicity using an insertional mutagenesis method, such as restriction enzyme-mediated insertion (REMI), transposon-mediated mutagenesis, targeted gene disruption, or gene silencing (Melania F. Betts and M. Alejandra Mandel, 2007). Over the past decade, *Agrobacterium*-mediated transformation (AMT) of fungi has been used extensively for insertion of reporter transgenes, random insertional mutagenesis ("T-DNA tagging"), targeted gene knockouts and gene silencing (REFs??) (Brown, 1998; Mullins, 2001; Ruiz-Diez, 2002; Weld, 2006). Fungal hyphae, spores and protoplasts can all be transformed using AMT (de Groot, 1998). Also, the T-DNA of AMT binary vectors can be engineered with a selectable resistance marker flanked by homologous fungal DNA and transformed into fungi, resulting in a higher frequency of homologous recombination than occurs with protoplast transformation.

An alternative insertional mutagenesis technique, REMI, has been used successfully to identify genes important for pathogenicity in many diverse filamentous fungi. Unfortunately, many of the mutations resulting from REMI can be either untagged or due to multiple insertions, which require a well-established genetic system to decipher. In contrast, most transformants resulting from AMT contain single T-DNA insertions, simplifying gene identification (E. D. Mullins, 2000). This AMT-based "T-DNA tagging" has been used in many fungal pathosystems of model plants, allowing significant advances in our undersatnding of the molecular bases of fungal pathogenicity (O'Connell, 2004).

Phoma medicaginis is an appropriate fungus for insertional mutagenesis since it is easy to culture and maintain stably in the laboratory, shows very short generation time, has monokaryotic (uninucleate) hyphae and conidia, and is easy to transformation with AMT (REFs). In this study, a novel AMT complementation vector (pCAM-NAT) based on

nourseothricin resistance will be constructed and used to complement two previously identified, T-DNA-tagged cultural mutants of *P. medicaginis*: one with slow growth and stunted pycnidia and another with non-melanized, hyaline pycnidia. In the first mutant, a gene predicted to encode a poly-A RNA polymerase (PAP) is disrupted by a single T-DNA insertion. In addition to complementation, a PAP deletion mutant will be generated by homologous recombination using AMT, and the resulting phenotypes characterized. The complementation of the tagged PAP and recapitulation of the mutant by gene deletion would functionally corroborate this gene's involvement in growth and asexual reproduction. In the second mutant, the sole T-DNA is inserted equidistant between two predicted genes, a serine-threonine protein kinase and a conserved gene of unknown function. The expression of these two flanking genes will be tested by reverse transcription (RT) PCR in the mutant and wild type strains and the altered gene complemented using AMT with pCAM-NAT.

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CHAPTER II

LITERATURE REVIEW

Medicago species and disease

The members of the genus *Medicago* L. (56 species) play important roles in Mediterranean flora and in applied botany(Lesins and Lesins, 1979; Rouxel and Balesdent, 2005). Alfalfa (*Medicago sativa* L.) is the dominant forage legume with worldwide economic importance. Alfalfa is a perennial that survives as numerous ecotypes adapted to growing throughout the world and is cultivated on more than 33 million hectares in USA and in Europe (Michaud et al., 1988). On average over the past 10 years, the United States annually produced ~66 million metric tons alfalfa (hay), valued at $7.8 billion (NASS-USDA, 2011). Over the same time in Oklahoma, about 1 million metric tons of alfalfa were harvested annually from ~135 thousand hectares, valued at $126 million (NASS-USDA, 2011).

The genus *Medicago* includes several annual medics, such as *M. truncatula* (barrel medic), *M. polymorpha* (burr medic) and *M. scutellata* (snail medic), which are employed as cover crops, short season forage crops, and weed-suppressing smother crops (Fisk et al., 2001). *Medicago truncatula* has also been studied extensively as a model

legume, along with *Lotus japonicus*. The genomes of most *Medicago* spp. are diploid or tetraploid with a basic haploid complement of 8 chromosomes (n = 8) (Lesins and Lesins, 1979).

As do all legumes (i.e. members of the Fabaceae), *Medicago* spp. form mutualistic symbioses with nitrogen-fixing rhizobia, including *Sinorhizobium meliloti* and *Sinorhizobium medicae*, and phosphorus-absorbing arbuscular mycorrhizal (AM) fungi (e.g. *Glomus* spp.). The rhizobia induce root nodules, where these bacteria fix atmospheric nitrogen into organic forms exchanged for photosynthates from the plant host (Long, 1989).

Model legume, *Medicago truncatula*

Medicago truncatula is an established model plant for legume biology (Cook, 1999). It possesses a small, diploid genome of about 500 Mbp, is self-fertile with abundant seed production, and a rapid generation time (Cook, 1999). The extensive genetic characterization of *M. truncatula*, the sequencing and EST annotation of its genome and the production of the Medicago Affymetrix GeneChip (Tesfaye et al., 2006) have made *M. truncatula* an attractive model plant system, comparable to *Arabidopsis thaliana*. However, it is the tractable symbiotic interactions with rhizobia and AM fungi that critically distinguish *M. truncatula* from Arabidopsis, which, as a crucifer, lacks either of these mutualisms.

Many mutants of *M. truncatula* in this symbiosis have been generated by different methods, such as ethyl methanesulfonate (EMS) (Penmetsa, 2000), gamma irradiation (Sagan, 1995a, 1998), and insertional mutagenesis (REF). Many EMS mutants altered in nodulation, like *dmi* (3 loci, doesn't make infection), *sunn* (supernumerary nodulation mutant) and *skl* (sickle) have been generated (Schiestl, 1991; Catoira, 2000). Complementary, several mutants of S*innorhizobium meliloti* altered in

nodulation and nitrogen fixation were generated by gamma irradiation (Sagan, 1995b). Recently, T-DNA or transposon based insertional mutagenesis have been used to generate mutants of *M. truncatula* with tagged loci (d'Erfurth, 2003; Schnabel, 2003). However, the T-DNA tagging method was inefficient in generating mutants compared to *Tnt1* retrotransposon-tagging, which has been used to effectively generate many mutants of *M. truncatula* (d'Erfurth, 2003; Tadege, 2005).

Numerous expressed sequence tags (ESTs) databases for *M. truncatula* have been developed (www.medicago.org). These include ESTs from different tissues of *M. truncatula* during many developmental stages of symbiosis and interactions with plant pathogens such as *Phoma medicaginis*, *Phytophthora medicaginis* Hansen & Maxwell, *Meloidogyne incognita* Kofoid & White, and *Colletotrichum trifolii*. Similarly, proteomic profiling of stem, leaf, seed, roots, and flower tissues, and cell suspensions of *M. truncatula* identified approximately 37,000 genes. (Bonnie S. Watson, 2003).

Spring black stem caused by *Phoma medicaginis* Malbr. & Roum.

Taxonomy of *Phoma medicaginis*

P. medicaginis is a part of the phylum Ascomycota, class Dothideomycetes, and order Pleosporales (Balesdent, 2005). This fungus displays similar phylogenetic affinity to both families, *Leptosphaeriaceae* and *Pleosporaceae* and has named *P. herbarum* var.

medicaginis Westend. ex. Rabenh., *P. medicaginis* Malbr. & Roum., *Diplodinia medicaginis* Oud., and *Ascochyta imperfecta* Peck. *P. medicaginis* var. *medicaginis* is the final name to distinguish from the var. *pinodella* (Morgan-Jones and Burch, 1987).

The genus *Phoma* Fr. has separated by 9 different sections to identify species: *Phoma, Heterospora, Paraphoma, Peyronellaea*, *Phyllostictoides, Sclerophomela, Plenodomus, Macrospora,* and *Pilosa*. The teleomorphs of the genus *Phoma* includes *Didymella* Sacc., *Leptosphearia* Ces. & De Not., *Mycosphearella* Johanson, and *Pleospora* Rabenh. ex Ces. & Des Not. The conidia shape, size, and septation and pycnidial cell wall structure are considered to identify *Phoma* species (Boerema, 2004a). *P. medicaginis* var. *medicaginis* belongs to *Phyllostictoides* displaying thin-wall pseudoparenchymatous, and ostiolate pycnidia and unicellular conidia *in vitro*, but septa *in vivo*. *Didymella* is the only teleomorph in this section. Chlamydospores is hardly to be formed.

Systematics of *Phoma medicaginis* and related fungi

Spring black stem and leaf spot is caused by *Phoma medicaginis* Malbr. & Roum var. *medicaginis* and *P. medicaginis* var. *macrospora* Boerema, Pieters & Hamers in alfalfa (Boerema, 2004b). *P. medicaginis* var. *macrospora* produces larger 'macroconidia' (up to 4× var. *mediciginis*) with 1 to 3 septa at low temperatures. At room temperature, the differences in conidial size and septation are not distinguishable between the two varieties. Also, *P. medicaginis* var. *macrospora* can be more virulent on alfalfa

than *P. medicaginis* var. *medicaginis* (REF#??). However, phylogenetically these two varieties are not separable and thus, may be synonymous within the family Didymellaceae (Aveskamp et al., 2010) (Aveskamp et al. 2010, Studies in Mycology 65).

Morphologically, *P. pinodella* (L.K. Jones) Morgan-Jones & K.B. Burch, formerly *P. medicaginis* var. *pinodella*, resembles *P. medicaginis*. However, *P. pinodella* infects pea and red clover, but hardly infects *Medicago* spp. (REF#??). Additional cultural characteristics distinguishing *P. pinodella* from *P. medicaginis* include *P. pinodella*'s frequent sectoring in culture, formation of crystals on malt extract agar (MEA) and production of chains of globose single-celled chlamydospores (White and Morgan-Jones, 1987). Phylogenetically, *P. pinodella* has been synonymized with *P. sojicola*, causal fungus of a soybean leaf spot (Irinyi et al. 2009 Mycol. Res.), and is also considered closely related to *Didymella pinodes* (anamorph *Ascochyta pinodes*). The anamorph genus, *Peyronellaea* Goid. Ex Togliani has been proposed emended by Aveskamp, Gruyter & Verkley to include *P. pinodella* and *A. pinodes* (Aveskamp et al., 2010).

Distribution and host range

Phoma medicaginis has been recorded around the world, on every continent except Antarctica. Spring black stem was first reported in 1908 in New York (Stewart, 1908) and can be found in Idaho, Washington, Kentucky, Colorado, Kansas, Minnesota, Nebraska, and Oklahoma (Farr et al., 2007).

P. medicaginis has been found in legumes such as *Arachis hypogaea* L. and *Glycine max* L. Merr. in Brazil, *Cicer arietinum* L., *Dolichos uniflorus* Lam., and *Pongamia pinnata* (L.) Pierre in India, *Glycine ussuriensis* Regel & Maack. In Zambia, *Medicago* spp. (worldwide), *Melilotus* spp. in China and U.S. and *Trifolium* spp in China, U.K. and Australia (Farr). It has also been reported as pathogen in non-legumes, *Brassica* spp. in Canada, *Capsicum annum* L. in Brazil and *Pennisetum clandestinum* Hochst. ex Chiov. In New Zealand (Farr). Even though *P. medicaginis* has wide host rage, it needs to re-evaluate reported comprehensive series of host with well-characterized isolates inoculated.

Symptoms and life cycle

The foliar disease, spring black stem, occurs with varying severity in nearly all cultivated alfalfa and has been implicated in the declining hay yields of older (>3 years old) fields (Kernkamp, 1953; Mead, 1953; Graham, 1972; Rodriguez, 1987; Barbetti, 1995a). The causal fungus *P. medicaginis* can infect and survive on every part of the alfalfa plant, including, notably, the seed, on which it can be disseminated. *P. medicaginis* can survive in the soil on plant debris as pycnidia, mycelia or chlamydospores, all of which can act as primary sources of inoculum.

The most common symptom on infected plants is smooth, black discoloration on the stems near the crown. As the disease become more severe, black spot on the stem advance up the stem. Stipules on infected stems wilt and turn brown. The disease is most severe in the spring, resulting in dead tissues on which the pathogen fruits heavily.

Pycnidia can develop on all infected host tissues and exude conidia in a mucilaginous matrix in humid conditions. Conidia are disseminated to healthy host tissues by rain and wind and act as the secondary inoculum during the spring and early summer. Older leaves within the canopy are more susceptible to infection by *P. medicaginis* (Valleau; Peterson, 1941). Other foliar pathogens and diseases of alfalfa include *Pseudopeziza medicaginis* causing common leaf spot and *Leptosphaerulina trifolii* causing Lepto leaf spot, and produce similar symptoms to spring black stem (Cormack, 1945b).

Biology of *Phoma medicaginis*

A. Growth *in vitro*

Colony types have been studied using different types of media, such as potato dextrose agar, malt extract agar, and oat meal agar. The form of colonies on these media is wavy, concentric, and irregular margins. On malt agar, aerial mycelium and crystals is well developed and byroid, dendritic, and brefeldin A are formed after 2 or 3 weeks (Noordeloos et al., 1993; Palumbo et al., 1998). The fungus grows as white colonies in the beginning, and then show olive-green color with white buff margins. The matured colonies become sector and aerial hyphae are fallen to the surface of media. The growth of fungus is favored between 21-24℃. The pycnidia are most developed between 21-27℃ under light (Morgan-Jones and Burch, 1987). The growth of fungus retards at 9℃, increases until 21℃, and stop at 33℃ on PDA. The favored temperature for production

of pycnidia is between 21℃ and 27℃ (Peterson and Melchers, 1942). The formation of pycnidia is more enhanced by nitrate in the media than ammonia (Chung and Wilcoxson, 1971).

B. Reproduction of *Phoma medicaginis*

P. medicaginis on alfalfa only reproduces using pycnidia forming pycnidiaspores in nature, whereas the other pathogen causes alfalfa disease including *Pleospora* spp., *Leptosphaeria* spp. And *Ophiobolus* spp. reproduces pseudothecia on alfalfa stem during overwinter (Johnson and Valleau, 1933; Cormack, 1945a). The fungus is haploid and conidiogenesis is formed in pycnidia. The pycnidiospores are single-celled, occasionally showing septae, and hyaline that exuded from pycnidia with pink mucilage. Chlamydospores are only found in matured culture (Johnson and Valleau, 1933; Schenck and Gerdemann, 1956).

C. Host-pathogen interaction

The interaction of between *P. medicaginis* and alfalfa has not been studied in detail despite spring black stem on alfalfa was first found in 1990s. The pathogen is able to reduce seedling emergence due to damping off when inoculating with mycelia (Schenck and Gerdemann, 1956). The symptoms appeared on stems and leaves after spray inoculation of blended mycelium to whole plants. In the beginning of penetration, hyphae are able to enter directly or through stomata. During penetration, the pathogen does not develop appressoria. Once the pathogen colonizes inside the host, the symptoms

become severe. The hyphae develop intercellular and intracellular space in the host cells and are able to grow intracellular dead tissues. The vascular tissues in old stems are free to infect by pathogen and it grows only cortical tissues. Young leaves and alfalfa are more susceptible than old ones. Pycnidia can be observed under the epidermis of stems and dead tissues.

D. Secondary metabolites of *Phoma* spp.

Phoma sp. produce numerous biological compounds *in vivio* and *in vitro*, but several secondary metabolites have been isolates in *P. medicaginis*. Several *Phoma* sp. including *P. medicaginis* secret the macrocyclic lactone brefeldin A (BFA) (Noordeloos et al., 1993). The fungal BFA has been studied in protein trafficking in the plant cell pathway, especially Golgi apparatus. Additionally, it inhibits the transport of protein from endoplasmic reticulum (ER) to the Golgi apparatus and causes the disorganization of Golgi in various plant cells (JIANG, 2007). The hindrance of carrying protein is caused by ER-Golgi hybrid compartment due to BFA treatment (Nebenführ et al., 2002). Once BFA treats the sycamore maple (*Acer pseudoplatanus*) cells, enlargement of ER, accumulation of cisternae, and increasing number of vesicles containing large amounts of xyloglucan (XG) are observed. All these effects are disappeared in 2 hours by elimination of BFA (Driouich et al., 1993). Generally, plant infected by *P. medicaginis* shows symptomless or signs including pycnidia on the limited lesions. In the moist incubation, the growth of pathogen promotes within 9 days on dead tissues of infected plant. These infected tissues contain brefeldin A (1.7 µg/g). Also, this production is observed on cultured media (20 mg/l) and inoculated *M. sativa* stems (3 mg/g). Brefeldin A inhibits

spore germination and growth of epiphytic fungi in the same phyllosphere. This metabolite may use for inhibiting competing fungi while the pathogen become saprotropic fungi from endophyte during life cycle (Weber et al., 2004). Brefeldin A is also gemerated by *Alternaria* spp and *Penicillium*. spp. This toxin was first characterized in *Penicillium decumbens* Thom (Singleton et al., 1958; Betina et al., 1962). The numerous other metabolites are observed in *Phoma* sp. and closely related Pleosporalean fungi such as sirodesmin, AM-toxin, T-toxin, and Ptr ToxA .

Management of spring black stem and leaf spot

Spring black stem and leaf spot caused by *P. medicaginis* has managed by various practices such as resistance cultivars, spraying fungicides, seed treatments. Varieties of resistance to *P. medicaginis* have been shown in annual spp (Barbetti, 1987). The severity of disease and rate of symptom development depends on temperature and humidity. Variation of resistance in *P. medicaginis* has been shown in *Medicago* spp. (Renfro, 1959; Barbetti, 1987). Barbetti (1987) conducted a test of range of species under controlled conditions resulted in the presence of large differences among cultivars in the same species. The best species of *Medicago* showing disease resistance is *Medicago murex*. The result of test for resistance to phoma leaf disease and stem disease on end of seasons is that M. *murex* cv. Zodiac and SAR 3490, *M. tornata* SA10012, *M. truncatula* cv. Parabinga, *M. murex* cv. Zodiac, SAR 3490, and 87F01.48; *M. sphaerocarpos* cv. Orion (SEP 29.1), SEP 29.2, and SEP 30.3; and *M. tornata* cv. have been shown most

resistant to disease. Planting young plant is important for disease severity. Usually, over 10 week plant become more susceptible than 1-4 week plant (Barbetti, 1995b).

In 1950s, applications of fungicides have been tested by several groups and they suggested that ziram, zineb, nabam and ferbam are not effective on spring black stem. However, benomyl, carbendazim, flutriafol, propicanazole or triadimefton show about effective controlling spring black stem and leaf spot with resistant cultivars. They also recommended that planting resistant cultivars along with non-host rotation is the best strategy for disease control (Barbetti, 1898; Barbetti, 1990).

Transformation of fungi

In order to investigate the role of genes in the filamentous fungi, transformation have been played dominant role through the gene disruption. This transformation can be used to determine the difference of phenotype between wild-type and mutants through the random insertional mutagenesis and targeted mutagenesis. In the case of random mutagenesis, genes in fungi are disrupted by selectable marker, resulting in defective genes using the various methods such as transposon mutagenesis and REMI, restriction enzyme mediated insertion. These insertional genes allow to identify the function of gene by sequencing. With targeted mutagenesis, double-crossover, homologous recombination occurs in the wild-type fungi through transformation result in knockout gene. The various methods of transformation have been used in making a mutagenesis. Many tools of transformation require the protoplasts which are made by cell wall degrading enzyme to

insert the DNA into host fungi. After making the protoplast, PEG, REMI, particle bombardment, electroporation and AMT are used for deliver transgene into fungal cell's nucleus. For transformation using PEG, polyethylene glycol, incubate for 10-50 min at room temperature in the high concentration of PEG.

Agrobacterium- mediated transformation (ATMT)

Agrobacterium genus can develop external galls and tumors along the crown of the stem of plants like tobacco. They are unique disease among phytopathogenic bacteria involving the transfer DNA called T-DNA into plant genomic DNA randomly. It has allowed field of genetic engineering to produce numerous transgenic plants and fungi. In the beginning of infection step, the bacterial cells make cellolose filamentous which attach the surface part of wounded host cell. The tissues of tumor provide the unique amino acids (opines) as carbon and nitrogen sources. T-DNA located in tumor-inducing (Ti) plasmid that includes the T-DNA itself, 25 bp imperfected repeats, and ~35 *vir* genes. The each expressed by *vir* genes achieve the transferring T-DNA to the nucleous of the host cell.

Selectable Marker

Various selectable marker genes have been used for selection of fungal transformation(Fincham, 1989). Usually, auxotrophic markers are applied to transforming diverse filamentous fungi. The other way is to use niaD gene of *A. nidulans* and pyrG or pyr-4 genes can be complemented to generate mutants with source of uracil. Also, Antibiotics resistance genes in bacteria such as hygromycin phosphotransferase (*hph*), bleomycinbinding protein (*ble*), benomyl-resistant Y-tubulin (*tub*) and phosphinothricin acetyltransferase (*bar*) are employed to select the transformed filamentous fungi.

We have already mutants that are disrupted into genomic DNA in *Phoma medicaginis* by T-DNA using AMT. These mutants showed to resistance to hygromycin B. In order to generate complementation for Koch's postulation, we should find alternative resistance gene which is not exhibit same resistance to hygromycin B. The nourseothricin acetyltransferase (nat1) as alternative marker is applicable for complementation vector. Several yeast species such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Candida albicans* and *Cryptococcus neoformans* (Goldstein and McCusker 1999, McDade and Cox 2001, Hentges et al. 2005, Shen et al. 2005) have been transformed using nat1 from *Streptomyces noursei* (Krügel et al. 1993).

Poly (A) RNA polymerase *cid* 13 gene

The canonical Poly (A) polymerase (PAP) is indiscriminately involved in polyadenylation of all mRNA. Recently, non-canonical PAPs act in the nucleus and

cytoplasm on regulation of specific RNAs (Norbury, 2006). The non-canonical PAPs genes have been reported six (cid like genes) in the fission yeast (table 1.) and 2 (*trf*4 and *trf*5) in the budding yeast (Rebecca L. Read, 2002). Caffein induced death aupressor (Cid)13 is located in nucleus and cytoplasm and involved in maintaining dNTP pools through ribonucleotide reductase (RNR) (Norbury, 2006). Many proteins are involved in managing fidelity of genome duplication in eukaryotic cells such as RNR that synthesize dNTPs into DNA. DNA polymerase with abundant and stable dNTPs is required for replication integrity. Appropriate supplying dNTPs is achieved using regulation of RNR. In the early studies, Cid13 is considered as cytoplasmic PAP; however, recent protein localization study suggested that Cid13 locates to the both nucleus and cytoplasm (Matsuyama A, 2006). A *cid*13 deleted mutant shows highly sensitivity to hydroxyurea (HU) in *Schisaccharomyces pombe* because HU affect negatively DNA synthesis by inhibiting RNR, resulting in decline of ribonucleotide to deoxyrebonucleotides.

Table 1. Poly (A) polymerase enzymes (PAPs) in *Sz. Pombe*

PAP	Localization	Known orthologues	Function
Pla 1	Nuclear	Pap 1p (*S.c.*), PAP (*H.s*)	Essential mRNA 3' processing enzyme
Cid 1	Cytoplasmic	CID-1 (*C.e.*)	Checkpoint response to replication block
Cid 11	Nuclear and cytoplasmic	?	?
Cid 12	Nuclear and	RDE-3 (C.e.)	For RNAi-dependent transcriptional

	cytoplasmic		silencing
Cid 13	Nuclear and cytoplasmic	?	Maintains dNTP pools via RNR
Cid 14	Nuclear and cytoplasmic	Trf4/5p (*S.c*)	Targets RNAs for exosome-mediated degradation
Cid 16	Mitochondrial?	hmtPAP (*H.s.*)?	Mitochondrial RNA processing?

S.c., Saccharomyces cerevisiae; *H.s.*, Homo sapiens; *C.e.*, Caenorhabditis elegans.

Melanin of phytopathogenic fungi

Melanin is dark pigmented polymers of phenolic or indolic monomer. These macromolecules form complex with protein or often carbohydrates. Many phytopathogenic fungi produce melanin involving in pathogenesis because it provides surviving structure to endure unfavorable environmental conditions during life cycle. These polymers are generally associated with biological and inorganic molecules. Its chemical characteristic is black and brown biopolymers, insolubility in cold or hot water and organic solvents, resistance to degradation concentrated acid, and oxidizing agents such as hydrogen peroxide (Butler, 1998; Henson, 1999). Fungi synthesize three different kind of melanin, L-dihydroxyphenylalanine (DOPA), glutaminyl-3,4-dihydroxybenzene (GDHB), and 1,8 dihydroxynaphthalene (DHN). Some mushroom species produce DOPA or GDHB melanin, whereas DHN is naturally formed by hyphae (Hegnauer H, 1985; Miranda M, 1992). DOPA mealnins have been studied in human melanin since this

monomer is involved in skin cancers and brain disorder (Parkinson's disease). It has been controversial that mushrooms synthesize DOPA mealnins that are produced by phenoloxidase (Prota, 1992). The *Tuber* genus forms DOPA melanin. The fungus rarely synthesizes monomer melanin at the cell wall, whereas it forms the melanin from exogenously supplied substrates (Butler, 1998). The cell wall melanin of *Agaricus bisporus* is developed by precursor GHB and the originate of melanin from teliospores of the *Ustilago maydis* is catechol melanin (Piatelli, 1965; Rast, 1980). The pathway of DHN melanin is well characterized in fungi as polyketide melanin. The beginning molecule of DHN melanin pathway is 1,3,6,8-tetrahydroxynaphthalene. It becomes 1,3,8-tri hydroxynaphthalene (THN) through dehydration reaction. Next step is THN is converted to DHN with second dehydration reaction, and then it is polymerized to DHN melanin (Butler, 1998).

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CHAPTER III

CONSTRUCTION OF AGROBACTERIUM-MEDIATED TRANSFORMATION VECTOR USING ALTERNATIVE SELECTABLE MARKER, NOUSEOTHRICIN RESISTANCE

Abstract

Phoma medicaginis causes significant quality and yield losses to alfalfa and annual medic crops worldwide. *Phoma medicaginis* also infects the model legume *Medicago truncatula* and is genetically transformable using *Agrobacterium tumefaciens* mediated transformation (ATMT). Using ATMT, T-DNA tagged mutants altered in pycnidial (asexual fruiting structure) morphology were identified. To verify the involvement of the disrupted gene, the mutation must be replicated through targeted gene replacement (knockout) and all mutants complemented with the wild type gene. This chapter focuses on the development of an ATMT vector with an alternative selectable marker that can be used for complementation. Since tagged mutants contain hygromycin resistance as a selectable marker, another dominant marker, nouseothricin (NTC) acetyltransferase (*nat1*), was used to construct a novel ATMT vector. The wildtype *Cid13* gene was then cloned into this vector and used to complement the disrupted *cid13*

gene in the mutant P265. Transformations with the empty complementation vector, pCAM-Nat, demonstrated it effectively complemented previously T-DNA tagged hygromycin resistant mutants. Our results suggest pCAM-Nat should be useful for future

complementation of other fungal mutants, which is essential for testing the function of genes.

Introduction

Agrobacterium tumefaciens-mediated transformation (ATMT) has been widely used for transformation of plants and fungi and results in the introduction of transfer DNA (T-DNA) into host genomes (Hellens et al., 2000). In order to transform the filamentous fungi, several marker genes have been used for selection of transformants (Lemke, 1995). The hygromycin phosphotransferase (*hph*) gene, which confers hygromycin B resistance, is the most common selection marker for the transformation of filamentous fungi because it is effective and useful in many fungal systems. The other selection markers confer resistance to phleomycin, sulfonylurea, nouseothricin, bialophos, carboxin, blasticidin S, and benomyl (B., 2002; Chung KR, 2002; Fitzgerald A, 2004; Temporini ED, 2004; Wirsel SGR, 2004). Among these selection markers, the *nat1* gene was used, which confers resistance to nourseothricin. This *nat1* gene encodes the nourseothricin acetyltransferase of *Streptomyces noursei* (Krügel, 1993) and has been employed successfully for transformation of yeast species such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe, Candida albicans* and *Cryptococcus neoformans* (Goldstein, 1999; McDade, 2001; Hentges, 2005; Shen, 2005). The *nat1* gene has been reported as an effective selectable marker for transforming filamentous fungi including *Acremonium chrysogenum* and *Sordaria macrospora* (Hoff, 2006).

In this study, an ATMT binary vector for fungal transformation with *nat1*, pCAM-Nat1, was constructed, and *A. tumefaciens* AGL-1 carrying this vector was used for complementation of the T-DNA tagged mutants of *P. medicaginis*, P265 and P1A17, through ATMT. ATMT was conducted with optimal number of fungal spores. Complementation mutants were selected on yeast peptone sucrose (YPS) containing nourseothricin (50 μg/ml).

Experimental Approach

Fungal cultures: Wild type and P2 and hygromycin resistant mutants, P265 (Table 1) was cultured on Petri plates of YPS (0.1% yeast extract, 0.1% tryptone, and 0.1% dextrose, and 1.8% agar), SMM (0.5 g/l glucose, 0.7 g/l NH_4NO_3, 0.3 g/l $MgSO_4$, 0.3 g/l $KH_2\ PO_4$, 1.2 g/l K_2HPO_4 and 0.001 g/l each of $FeC1_3$, $CuS0_4$, $ZnS0_4$ and $MnSO_4$), and PMM (10 g/l sucrose, 2.4 g/l $NaNO_3$, 0.6 g/l $MgSO_4$, 2 g/l $KH_2\ PO_4$, 1.2 g/l K_2HPO_4 and 0.1 g/l each of $CaCl_2$ and NaCl) media. For transformation, wild types and mutants were incubated on MYPS (YPS containing 1.5% malt extract) at 18-20℃ under cool white fluorescent lights. Spores were collected by rubbing 7-10 d old cultures with a spreader in 3 ml of sterile milliQ water. Spore suspensions were calibrated using a hemocytometer and adjusted to $2x10^5$ spores/ml.

Bacterial cultures: The *E. coli* strain DH5α (20% glycerol stocks stored at −80°C) was cultured on LB (tryptone 10 g, yeast extract 5 g, NaCl 10 g and 1.5% agar) supplemented with 100 mg/L ampicillin and/or kanamycin to maintain plasmids with these selectable markers. Glycerol stocks (20%) of *A. tumefaciens* AGL-1 were stored at −80°C and

cultured on Agrobacterium minimal media (AMM; 2.05 g K_2HPO_4, 1.45 g KH2PO_4, 0.5 g NH_4NO_3, 1.5 g NaCl, 0.01g $CaCl_2$, 0.25g $MgSO_4$, 2.5 mg $FeSO_4$, 2 g glucose, 20 µl trace elements stock solution (Vogel, 1964)) at 28℃ for 3 d. Media for AGL-1 was amended with ampicillin (100 µg/ml) and kanamycin (100 µg/ml) to maintain the *recA* genotype and the binary vectors, pCB403.2 and pCAM-Nat1, respectively (Table 1).

Table 1. Fungal and bacterial strains and plasmids used in this work

Strains	**Genetic traits**[a]	**Relevant characteristics**
Phoma medicaginis		
P2	Wild-Type	
P265	Hyg^R, GFP, $G418^R$, *cid13*::*TDNA*	Fewer, smaller pycnidia and aerial hyphae
Agrobacterium tumefaciens		
AGL-1	*recA*::*bla*, Amp^R	Routine ATMT
ATMT plasmids		
pPTGFPH	Kan^R, Hyg^R, GFP, $G418^R$	T-DNA tagging
pBHt2-tdTom	Kan^R, Str^R, Hyg^R	Fluorescent protein
pCB403.2	Kan^R, $G418^R$	Gene deletion vector
pCB403.2 (*Δcid13*::*hph*)	Kan^R, $G418^R$, Δ*cid13*, Hyg^{R2}	Gene deletion of *CID13*, G418R counter

pCAM-Nat1	Kan^R, Ntc^R	Complementation vector
pCAM-Nat1(*PmCID13*)	Kan^R, Ntc^R, *CID13*	Complementation of *cid13*

[a] Genetic traits are as follows: Hyg^R = hygromycin B resistance (*hph*) , Kan^R = kanamycin resistance (nptIII), Str^R= streptomycin resistance (strA), $G418^R$ = G418 resistance (nptII) and Ntc^R = nourseothricin resistance (Nat1)

Construction of complementation vector: For complementation of T-DNA tagged mutants of *P. medicaginis* that have been disrupted using the selectable markers, *hph* and *nptII*, the P*trp*C-nourseothricin (*nat1*) gene from pD-Nat1 (FGSC) plasmid was used to replace the CaMV P*35S*::*hph* region of the binary vector, pCAMBIA 1300 (Kuck and Hoff, 2006) (Fig. 1). Using the error-correcting KOD DNA polymerase (EMD Millipore, Darmstadt, Germany), P*trp*C-Nat1 fragment was amplified with primers (Table 2) containing engineered *Bst*XI and *Xho*I sites, digested with each restriction enzyme and ligated into the pCAMBIA 1300 digested with the same enzymes. The resulting ATMT binary vector, designated pCAM-Nat1, was maintained in *E. coli* DH5α, purified and transformed into competent cells of *A. tumefaciens* AGL-1 using electroporation (Shen and Forde, 1989).

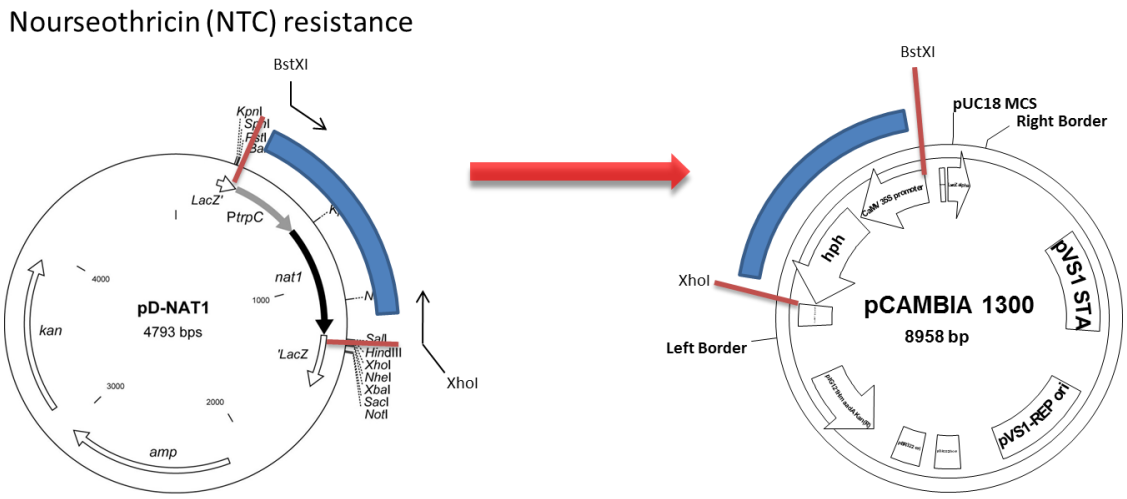

Fig 1. Construction of the binary vector pCAM-Nat1 used for complementation of T-DNA tagged *P. medicaginis* mutants.

Table 2. Primers used for complementation vector amplification.

Name	Sequence (5' to 3')	Tm	Description
BstXI-trpC-F	CGCCAACATGGTGGACAGAAGATGA	64.2	Amplify trpC-Nat1 fragment from pD-Nat1
XhoI-Nat1-R	GCGCCTCGAGGCAGGGGCAGGGCA	72.8	Amplify trpC-Nat1 fragment from pD-Nat1
pCAM1300-LB-R	TTTTGTGCCGAGCTGCCGGT	62.9	Confirm pCAM-Nat1
pCAM1300-BstXI-F	TTGCGCTCACTGCCCGCTTT	63.1	Confirm pCAM-Nat1
Nat-R	ATCGCCGGTGCGTTGACGTT	62.6	Confirm the complementation strains with BstXI-trpC-F primer

Fungal transformation: Single colonies of *A. tumefaciens* AGL-1 containing pCAM-Nat1 and AGL-1 containing pBHt2-tdTom (Codon Devices, Inc., Cambridge, MA; tdTomato sequence from (Shaner et al., 2004)) were cultured for fungal transformation in 5 ml of AMM supplemented with 100 mg/ml ampicillin and 100 mg/ml kanamycin in sterile 15 ml Falcon tubes, and incubated in an angled rack on a shaker at 28℃ for 3 d. Optical densities at 600 nm (OD600) were measured using a spectrophotometer. Bacterial cells were diluted to OD600 0.2 in virulence-inducing minimal medium broth (IMM; 2.05 g/l K_2HPO_4, 1.45 g/l KH_2PO_4, 0.5 g/l NH_4NO_3, 1.5g/l NaCl, 0.01 g/l $CaCl_2$, 0.25 g/l $MgSO_4$, 2.5 mg/l $FeSO_4$, 0.9 g/l glucose, 5.33 g/l MES, 5 ml glycerol, 20 ul trace elements stock solution (Vogel, 1964), 200 μM acetosyringone and appropriate antibiotics) and incubated overnight at 28℃, in 15 ml tubes shaking at 250 rpm. The OD600 cultures were adjusted to OD 0.2 with IMM broth. These virulence induced cultures were stored at 4℃ for 7 d.

The *P. medicaginis* spore suspension was diluted to a concentration of $2x10^5$ spores/ml using sterilized milliQ water. Then, 200 μl of the diluted spore suspension was mixed (1:1) with 200 μl of the induced *Agrobacterium* cultures. Two-hundred microliters of the 200 ul of spore-culture mixes were spread onto 47 mm nitrocellulose membranes (Fisher Scientific, Pittsburgh, PA) overlaid on a 60 mm IMM plates containing appropriate antibiotics and incubated at 20℃ in the dark for 2-3 d. Induced, vector-less AGL-1 was co-incubated with conidia as a negative control for each transformation.

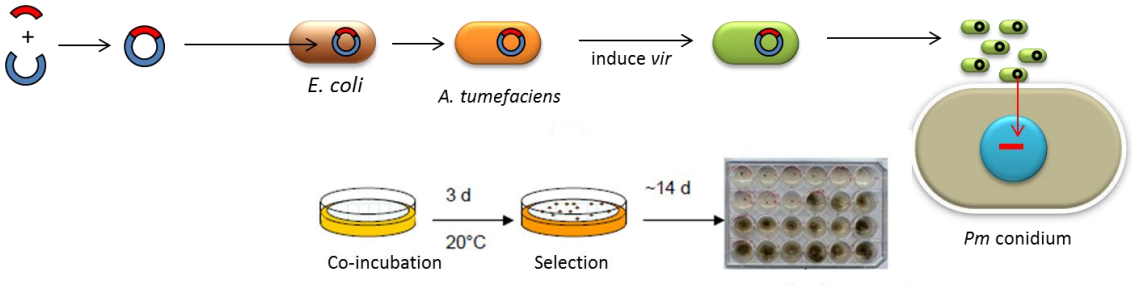

Fig 2. *Agrobacterium*-mediated transformation of *Phoma medicaginis*.

Transformants selection: After 2-3 d, membranes were transferred to 60 mm plates of YPS containing 50 µg/ml Ntc and 200 µg/ml timentin and 50 µg/ml cefotaxime for selection of Ntc resistant transformants and elimination of *Agrobacterium*, and incubated at room temperature in a dark. After 5-10d, the transformants penetrated the membrane and grew into the selective media. Hyphal-tips of each colony were transferred to individual wells of 24-well YPS plates containing 50 µg/ml Ntc. Spores incubated with the vector-less AGL-1 were unable to grow on the membrane selection plates.

Purification and stability of transformants: For purification of transformants, hyphal tips from single colonies of each transformants were transferred 3 times on 60 mm plates containing selective media. The stability of transformants was tested by transferring to YPS plates without antibiotics and then, transferred back to YPS with antibiotics. These transfers supported the assumption that successful integration of transgenes had occurred by amplified with each flanking DNA and antibiotic resistance gene. Each transformant was streaked on filter paper placed on the selective media. Once transformants were fully grown on the paper, the colonized papers were completely desiccated in dry oven and cut into several pieces. The pieces of colonized filter papers were put in sterile glass vials, sealed with parafilm, and stored at 4℃ in a storage box.

Isolate genomic DNA using Lithium acetate (LiOAc)-SDS: For quick and low-cost genomic DNA isolation from *P. medicaginis*, a LiOAc-SDS method was used to extract DNA from mycelia of the wild-types and transformants, according to the following modified procedure (Lõoke et al., 2011):

1. Pick an agar plug including agar of margin area of each *P. medicaginis* culture on YPS. Suspend plugs in 100 µl of 200mM LiOAc, 1% SDS solution.

2. Incubate for 5 minutes at 70℃.

3. Add 300µl of 95% ethanol, vortex.

4. Spin down DNA and cell debris at 15,000×g for 3 minutes.

5. Wash the pellet with 70% ethanol.

6. Dissolve the pellet in 100 µl of TE and spin down cell debris for 15 seconds at 15,000×g.

7. Use 1 µl of supernatant for PCR

Results

Construction and verification of complementation vector

In order to construct the complementation vector pCAM-Nat1, P*trpC-Nat1* was amplified from pD-nat1 using BstXI-trpC-F and XhoI-Nat1-R primers (Fig 3, lane 2). The complementation vector pCAM-Nat1 was constructed as described in the Materials and

Methods and the construct was verified by PCR using primers pCAM1300-LB-R and pCAM1300-BstxI-F, which amplified the T-DNA containing the P*trpC-Nat1* fragment (Fig 4, lane 2), which was smaller than the original P*35S-hph* containing product from pCAMBIA 1300 (Fig 4, lane 1). Also, this amplicon from the resulting pCAM-Nat1 plasmid was shown to be identical to the nouseothricin acetyltransferase gene in PZPnat1 by sequencing (Fig 5).

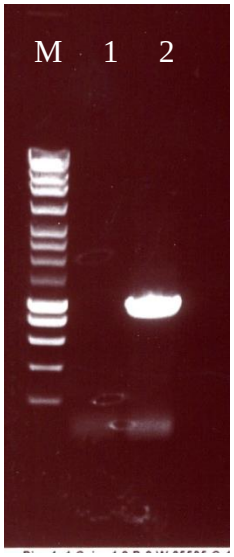

Fig 3. PCR amplification of P*trpC-nat1* fragment in pD-Nat1. Lane 1: negative control (water), lane 2: pD-Nat1 and M: 1 kb DNA ladder (Apex)

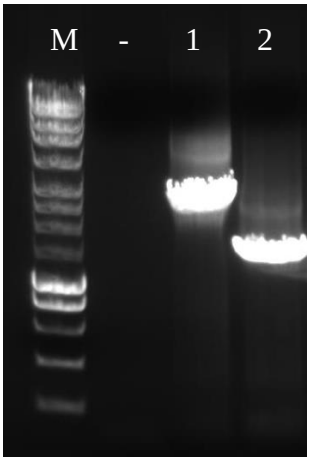

Fig 4. PCR analysis of replacing the selectable marker hygromycin resistance gene for the nouseothricin resistance gene. Lane 1: pCAMBIA 1300, lane 2: pCAM-Nat1, M: 1 kb DNA ladder and -: negative control (water)

>gb|AY631958.1| Cloning vector PZPnat1, complete sequence
Length=8492

Sort alignments for this subject sequence by:
E value Score Percent identity
Query start position Subject start position

Score = 886 bits (982), Expect = 0.0
Identities = 541/564 (96%), Gaps = 10/564 (1%)
Strand=Plus/Minus

```
Query  302   CAGGGGCAGGGCATGCTCATGTAGAGCGCCTGC---TCGCCGTCCGAGGCGGTGCCGTCG  358
             ||||||||||||||||||||||||||||||||   ||||||||||||||||||||||||||
Sbjct  1389  CAGGGGCAGGGCATGCTCATGTAGAGCGCCTGCCGCTCGCCGTCCGAGGCGGTGCCGTCG  1330

Query  359   TACAGGGCGGGTGTCCAGGCCGCAGAGGGTGAACCCCATCCGCCGGTACGCGTGGATCGCC  418
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1329  TACAGGGCGGGTGTCCAGGCCGCAGAGGGTGAACCCCATCCGCCGGTACGCGTGGATCGCC  1270

Query  419   GGTGCGTTGACGTTGGTGACCTCCAGCCAGAGGTGCCCGGCGCCCCGCTCGCGGGCGAAC  478
             ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  1269  GGTGCGTTGACGTTGGTGACCTCCAGCCAGAGGTGCCCGGCGCCCCGCTCGCCGGCGAAC  1210

Query  479   TCCGTCGCGAGCCCCATCAACGCGCGCCCGACCCCGTGCCCCCGGTGCTCCGGGGCGACC  538
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1209  TCCGTCGCGAGCCCCATCAACGCGCGCCCGACCCCGTGCCCCCGGTGCTCCGGGGCGACC  1150

Query  539   TCGATGTCCTCGACGGTCAGCCGGCGGTTCCAGCCGGAGTACGAGACGACCACGAAGCCC  598
             |||||||||||||||||||||||||||||| | ||||||||||| ||||||||||||||
Sbjct  1149  TCGATGTCCTCGACGGTCAGCCGGCGGTTCCACGCCGAGTACGAGATGACCACGAAGCCC  1090

Query  599   GCCAGGTCGCCGTCGTCCCCGTACGCGACGAAcgtccgggagtccgggtcgccgtcctcc  658
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1089  GCCAGGTCGCCGTCGTCCCCGTACGCGACGAACGTCCGGGAGTCCGGGTCGCCGTCCTCC  1030

Query  659   ccgtcgtccgattcgtcgtccgattcgtcgtcgggaACACCTTGGTCAGGGGCGGGTCC  718
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1029  CCGTCGTCCGATTCGTCGTCCGATTCGTCGTCGGGGAACACCTTGGTCAGGGGCGGGTCC  970

Query  719   ACCGGCACCTCCCGCAGGGTGAAGCCGTCCCCGGTGGCGGTGACGC-GAAGACGGTGTCG  777
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  969   ACCGGCACCTCCCGCAGGGTGAAGCCGTCCCCGGTGGCGGTGACGCGGAAGACGGTGTCG  910

Query  778   GTGGTGAAAGAACCCATCCAGTGCCCTCGATAGCCT-GGCATCCGCCCGGGACACCTGGT  836
             |||||| ||| |||||||||||| ||||||| |||| ||| ||| |||||||||| |||||
Sbjct  909   GTGGTG-AAGGACCCATCCAGTG-CCTCGATGGCCTCGGCGTCC-CCCGGGACA-CTGGT  854

Query  837   GCAGTACGCGT-AGCCCTGTCTTC  859
             || ||||  || |||| |||| ||
Sbjct  853   GCGGTACCGGTAAGCCGTGTCGTC  830
```

Score = 161 bits (178), Expect = 7e-36
Identities = 96/98 (98%), Gaps = 2/98 (2%)
Strand=Plus/Minus

Fig 5. Sequence analysis of the pCAM-Nat1 complementation vector

Confirmation of transformants:

The complementation vector, pCAM-Nat1, was used successfully to secondarily transform hygromycin resistant (Hyg^R) transformants, P1-A17 and P265, to NTC^R (Table 1), demonstrating that it could be useful for complementation. Three hundred eighty-one NTC^R transformants of P1A17 were generated through ATMT using pCAM-Nat1 (Table 1). Among the 381 transformants, 301 transformants were likely homologous recombinants since hygromycin resistance (Hyg^R) was lost. The remaining 81 transformants were presumed to be ectopic recombinants, as both were NTC^R and Hyg^R. In the case of P265 transformants, only presumed ectopic recombinants (Hyg^R/NTC^R) were recovered after ATMT with pCAM-Nat1. The transformation efficiencies of P1-A17 and P265, using the pCAM-Nat1 empty vector, were 0.002% and 0.0001%, respectively.

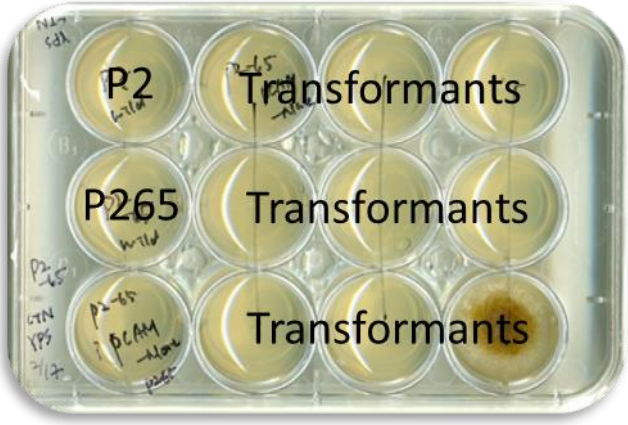

Fig 6. Nourseothricin resistant (NtcR) transformants that include complemented P265 candidates cultured on YPS containing NTC. Inhibited non-transformed wild type controls are also pictured (P2).

Table 3. Frequency of gene replacement using empty vector pCAM-Nat1 based on Ntc^R colony phenotypes.

Recipient mutant	Homologous	Ectopic
P1A17	301/381, 79%	80/381, 21%
P265	0/31, 0%	31/31, 100%

Complemented transformant strain P265C8 was verified by PCR to contain the T-DNA of pCAM-Nat1. The nouseothricin resistance gene was amplified with genomic DNA of complemented transformants and plasmid DNA of complementation mutants while wild type and original mutant strains were not amplified.

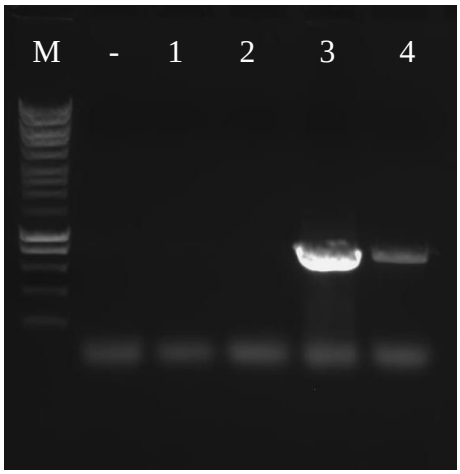

Figure 7. PCR analysis of nouseothricin resistance gene in *P. medicaginis* transformant Lane 1: P2, lane 2: P265, lane 3: pCAM-Nat1, lane 4: P265C8, M: 1 kb DNA ladder and -: negative control (water)

The original mutants (P265 and P1A17) were generated by pPTSGFP, while pBHT2 showed hygromycin B resistance as selectable marker (data not shown). For the selection of complementation transformants, another selectable marker, nouseothricin acetyltransferase (*nat*1) gene was considered to construct the complementation vector. These results demonstrated that the pCAM-Nat1 vector could be used for effective complementation of P265 and P1A17.

Discussion

The *nat1* gene was successfully used for transformation through ATMT of *Phoma medicaginis*. This observation is congruent with previous reports of ATMT and biolistic transformation of fungi such as *Penicillium chrysogenum* and *Cryptococcus neoformans* using *nat1* (Kim et al., 2009; Boer et al., 2013).

The efficiency of transformation using the complementation vector pCAM-Nat1 is close to that reported for other closely related filamentous fungi using ATMT (Mogensen et al., 2006). For example, 16.1 per 10^5 pycnidiospores of transformants were obtained with *Ascochyta rabiei* using an *A. tumefaciens* AGL1 strain, and 5-18 per 10^5 were produced with *P. medicaginis* using the *A. tumefaciens* LBA 4404 strain (Mogensen et al., 2006; Dhulipala, 2007). *Fusarium circinatum*, using *A. tumefaciens* strain AGL1, and *Aspergillus awamori*, using *A. tumefaciens* strain LBA1100, showed high transformation efficiency, producing 2-150 transformants per 10^5 and 10-90 transformants per 10^5 conidia, respectively. While *Aspergillus giganteus* (with LBA1100), *Coniothyrium minitans* (with AGL1), and *Botrytis cinerea* (with A.

tumefaciens strain LBA1126) displayed lower efficiency than *P. medicaginis* obtained using AGL1 (Mogensen et al., 2006).

While this result indicated that pCAM-Nat1 was a useful complementation vector to select transformants using an alternative selectable marker, ATMT with an empty vector was more effective to delete a selectable marker by homologous recombination. This result indicated that the empty vector might be used to remove a selectable marker from a mutant prior to experimental tests.

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CHAPTER IV

CHARACTERIZATION OF A NONCANONICAL POLY(A) RNA POLYMERASE GENE IN *PHOMA MEDICAGINIS*.

Abstract

Phoma medicaginis causes spring black stem and leaf spot of alfalfa (*Medicago sativa*) and annual medics, including the model legume *M. truncatula*, worldwide. *Phoma medicaginis* is genetically tractable using *Agrobacterium* mediated transformation (ATMT), with which T-DNA-tagged mutants altered in pycnidial morphology were generated. One mutant (P265) displayed fewer and smaller pycnidia and more aerial hyphae than the wild type. A single T-DNA disrupted a putative noncanonical poly(A) RNA polymerase gene, Pm*CID13*, which in yeasts interacts with ribonucleotide reductase (RNR). As in yeast mutants, P265 showed more sensitivity to hydroxyurea (HU), a RNR inhibitor. To functionally characterize Pm*CID13*, targeted ΔPm*cid13* mutants were created using a hygromycin selectable marker flanked by 1 kbp regions of Pm*CID13*. The ΔPm*cid13* mutants possessed morphological features similar to those of P265. The complementation vector pCAM-Nat1 (nourseothricin selection) was constructed and used to introduce full-length Pm*CID13* into P265 strain. Complemented P265 recovered wild type morphologies and often lost the original T-DNA due to

homologous integration. To our knowledge, this is the first *CID13* ortholog to be examined in a filamentous fungus.

Introduction

The genomes of numerous filamentous fungi have been sequenced and annotated (REFs?). In order to characterize the function of genes tentatively identified by homology-based annotations, several molecular methods must be available. First, a robust transformation system must be available. And ideally, fungi should be tractable to genomic manipulation through gene disruption or modification, including random and targeted mutagenesis, and genetic or transgenic complementation. Both mutagenesis and complementation are required to confirm a gene's function. In most cases, these general methods must be modified and optimized specifically for each fungal system studied due to the many variable and unique traits of each species(Weld et al., 2006)..

Agrobacterium tumefaciens-mediated transformation (ATMT) has been used for both random and targeted mutagenesis in filamentous fungi (Michielse et al., 2005). The high frequency of single insertional of T-DNA in transformants, resulting in simple identification of gene over that of restriction enzyme mediated integration (REMI) producing often multiple insertions (Mullins, 2001). Frequency and stability of homologous recombination depends on the method of transformation used in Serotype D strains of *Cryptococcus neoformans* (Davidson et al., 2000).Mutants that are generated by ATMT often contain a single T-DNA integration per genome, though multiple integrations also can occur occasionally. T-DNA insertion sites can be analyzed by inverse or TAIL-PCR to characterize flanking genomic DNA (Mullins, 2001; White, 2006).

In the previous studies, T-DNA tagged mutants of *P. medicaginis* were generated using ATMT (Dhulipala, 2007). Among these, pycnidial mutants were selected to further investigate. One of these, P2-65, produces smaller and fewer pycnidia and more aerial hyphae compared to wild-type (WT) (Dhulipala, 2007).

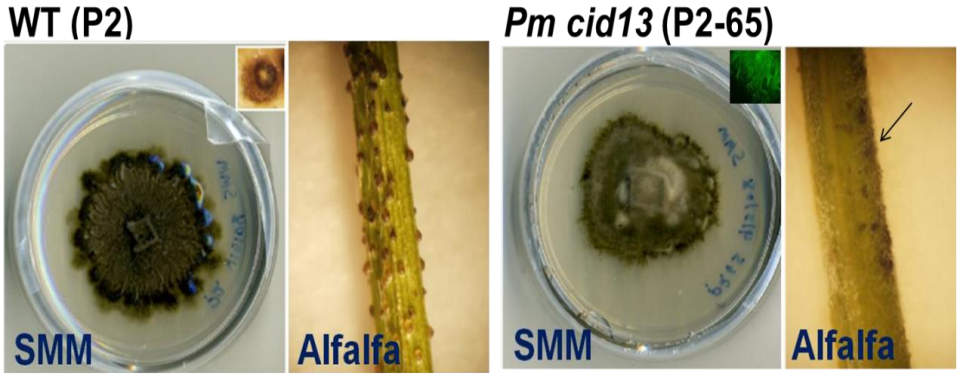


Fig 1. Morphological characteristics of WT strains, P2 and T-DNA tagged mutant, P2-65 on synthetic minimal media (SMM) plate, 14 day post inoculation (dpi) and alfalfa stems, 7 dpi. On alfalfa stems, P2-65 displays fewer and smaller pycnidia (arrow). P2 and P2-65 insets show wild type pycnidia and GFP fluorescence in hyphae, respectively.

Using an adaptor-ligated PCR approach (GenomeWalker, Clontech Laboratories, Mountain View, CA), Pm*cid13* gene of P2-65 was determined to be have been disrupted by a single T-DNA containing three genetic markers: hygromycin resistance (HPH), green fluorescent protein (GFP) and G418 resistance (NPTII) from the ATMT binary vector, pPTGFPH. A single T-DNA insertion in the genomic DNA of P2-65 was confirmed through Southern hybridization (Dhulipala, 2007). PmCID13 appears to encode a noncanonical poly(A) polymerase (ncPAP), similar to *Cid13,* first identified in *Schizosaccharomyces pombe* (Saitoh et al. 2002).

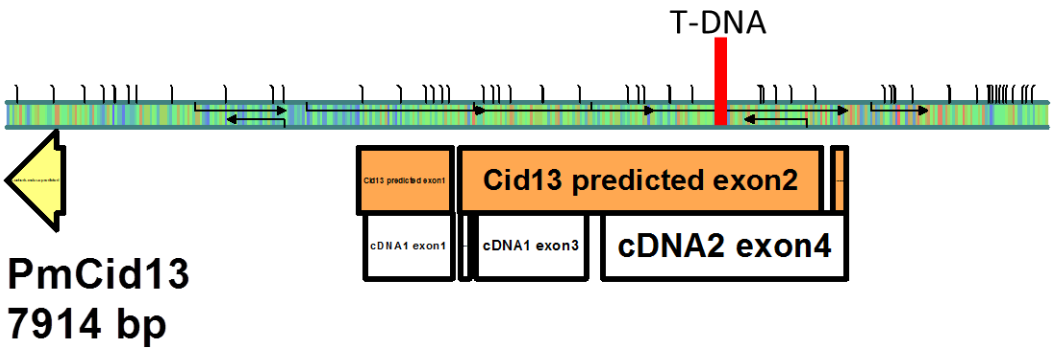
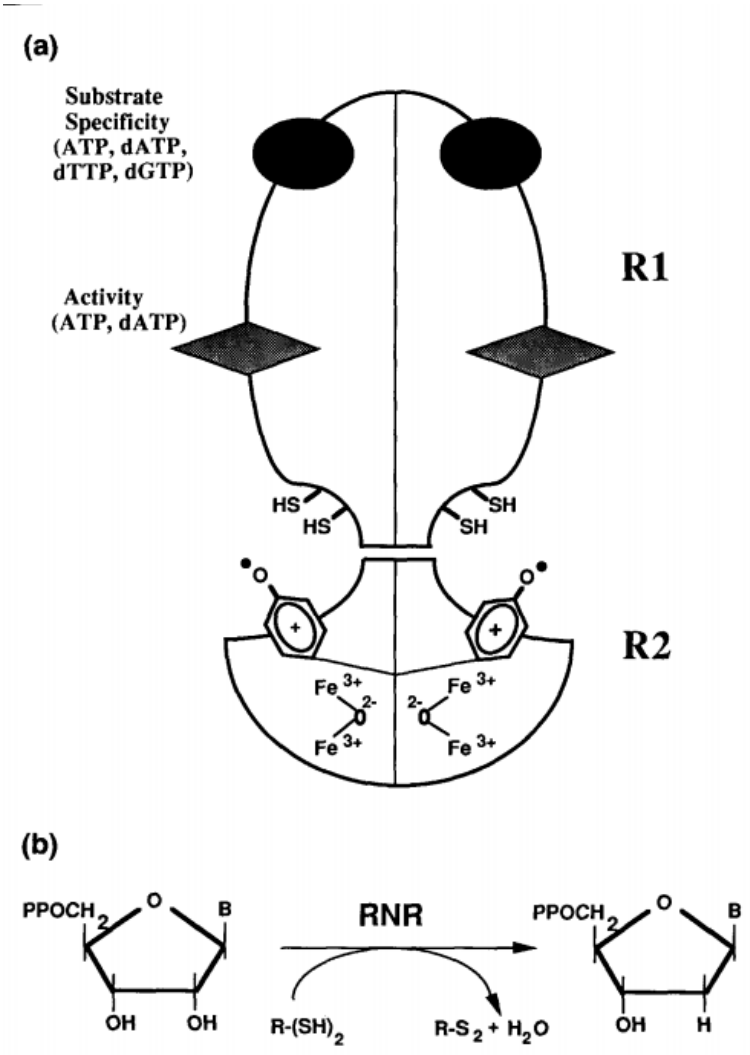


Fig 2. *Pmcid13* gene including predicted exons, conserved C-terminal region in filamentous fungi and T-DNA insertion site of P265

The process of transcription is highly complicated with mRNA undergoing many post-transcriptional processing steps including, capping, poly(A) adenylation, splicing, endo- and exonucleolytic trimming, and base modification (San Paolo et al., 2009). These steps ensure RNA quality and thus, protein synthesis and other cellular processes are maintained (Vanacova and Stefl, 2007). One of the posttranscriptional process involves the polyadenylation and deadenylation of mRNA (Goldstrohm and Wickens, 2008; Radford et al., 2008). The poly(A) tail is added by nuclear, canonical poly(A) polymerase (PAP) to the almost every premature RNA, and then cytoplasmic PAP manipulates the poly(A) length of specific mRNAs, altering their stability (Wickens et al., 2000; Edmonds, 2002) and translation levels (Jacobson and Peltz, 1996; Richter, 2000; Wickens et al., 2000). Non-canonical PAPs in the cytoplasm also add poly(A) tails to specific mRNAs resulting in an up regulation of expression, whereas canonical PAP catalyze polyadenylation in the nucleus (Wang et al., 2000). The first reported non-canonical PAPs in fission yeast *Schizosaccharomyces pombe* were found to be involved in the S-M phase. In fission yeast, RNA stabilization involves in six non-canonical PAPs: Cid1, Cid11, Cid12, Cid13, Cid14, and Cid16 (Stevenson and Norbury, 2006).

The budding yeast *Saccharomyces cerevisiae* has been found possess only two Cid1 family proteins, Trf4p and Trf5p as orthologous genes. Both Trf4p and Trf5p are located in nucleus whereas fission yeast Cid1 family localizes to both the nucleus and cytoplasm (Walowsky et al., 1999). The nuclear exosome complex degrades and directs the maturation of different RNA species, though purified exosomes from *Saccharomyces cerevisiae* show restricted functions (Mitchell et al., 1997). The yeast exosome is comprised of the Trf-Air1/2p-Mtr4p polyadenylation (TRAMP) complex, which includes the PAP Trf4p, one of two functionally redundant zinc-knuckle proteins, Air1p or Air2p, and the putative RNA helicase Mtr4p/Dob1p (LaCava et al., 2005; Vaňáčová et al., 2005; Wyers et al., 2005). In 2002, the non-canonical PAP, Cid13, was identified and characterized in fission yeast and found to add the poly(A) tail to *suc22* mRNA, which encodes the small subunit of ribonucleotide reductase (RNR) (Saitoh et al., 2002). RNR consists of two similar subunits: the large subunit R1, containing the regulatory and allosteric sites, and the small subunit R2, containing the active site and binuclear iron center (Fig. 2). This heterotetrameric enzyme synthesizes the deoxyribonucleotides (dNTPs) by catalyzing the ribonucleotides (Nordlund and Reichard, 2006). RNR is highly conserved and catalyzes the formation of dNTPs used in DNA replication and repair by supplying fidelity of dNTPs (Taipale, 2012).



Elledge et.al., 1992.

Fig 3. (a)The structure of eukaryotic ribonucleotide reductase (RNR). A dimer of large subunit R1 forms the allosteric and regulatory part. A dimer of small subunit R2 contains the catalytic sites, iron center and tryosyl free radical. (b) The reaction catalyzed by RNR.

The genes for the RNR subunits in *S. pombe* are encoded by *cdc22*, the R1 large subunits, and *suc22*, the R3 small subunits (Sarabia et al., 1993). The small subunit Suc22 is localized in the nucleus but relocates to the cytoplasm. Once S phase starts and the cell undergoes DNA damage affecting to cell cycle checkpoint activation, tetramerisation of

RNR occurs, producing dNTPs (Liu et al., 2003). The reduction of the ribonucleotides by RNR is triggered by generation of stable tyrosyl radicals requiring oxygen in the Fe-O-Fe center (Harder and Follmann, 1990; Nordlund and Reichard, 2006). Hydroxyurea (HU), which is used in cancer chemotherapy, inhibits the RNR activity by quenching the free radical (Harder and Follmann, 1990).

Previously, a T-DNA-tagged mutant of *P. medicaginis* P2 was generated and found to possess a disruptive integration in a ncPAP gene with similarity to SpCid13. The *cid13* gene (Pm*cid13*) has been highly conserved in ascomycetes based on phylogenetic analysis (Fig 3; Smith et al., 2009). P265 was generated using ATMT with vector pPTGFPH and its T-DNA encodes three marker genes encoding hygromycin and G418 resistance and constitutive green fluorescent protein (GFP) expression. The morphological characteristics of P2-65 are more white, aerial hyphae and smaller and fewer pycnidia compared to wild-type, P2.

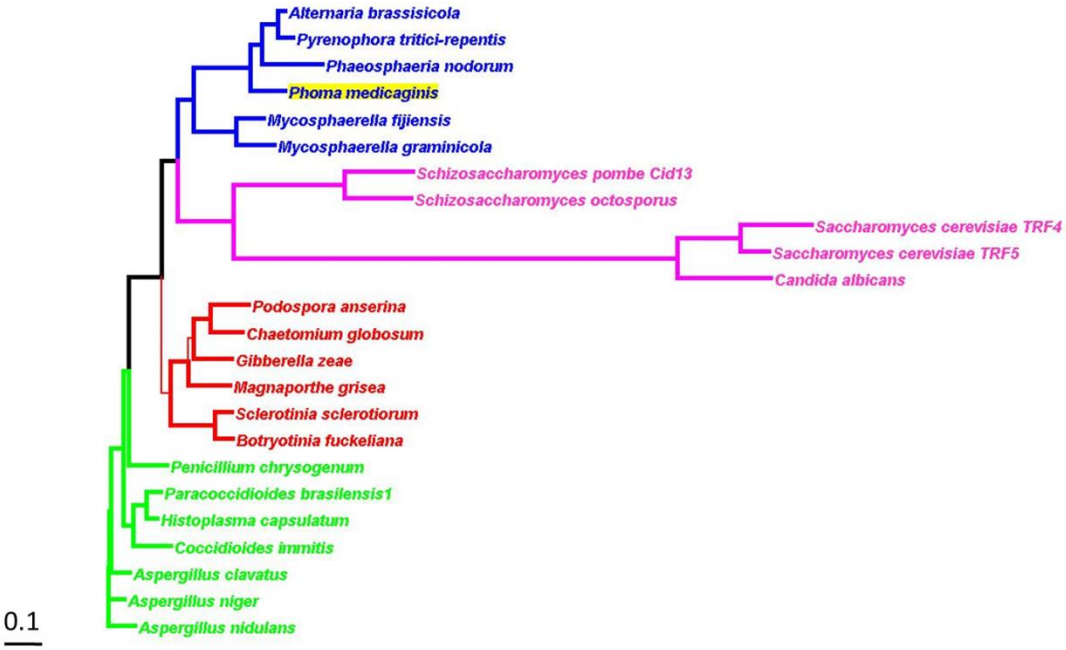


Fig 4. The phylogenetic tree of cid13-like protein (Smith et al., 2009)

The main objectives of this study are to

1. Construct ATMT vectors for mutant complementation using pCAM-Nat1 and for gene replacement (knock out) using pCB403.2
2. Complete the functional characterization of pycnidial mutant P2-65 of *Pho ma medicaginis*

Experimental Approach

Strains, media and culture condition: All strains of *P. medicaginis* were routinely cultured on YPS (0.1% yeast extract, 0.1% tryptone and 0.1% dextrose, and 1.8% agar)

plates. Liquid cultures of *P. medicaginis*, used for nucleic acid extractions, were grown as follows: Three 1 cm^2 agar plugs from a 7-14 day old YPS mycelial culture were added into a 2 ml screw cap tube containing three of 3 mm beads and one of 6 mm bead with 1 ml sterilized water and homogenized with a beadbeater (BioSpec Products, Inc. Bartlesville, OK) at maximum rpm for 20 sec. One ml of this suspension was inoculated into a tissue culture flask with vented caps (Corning, Randor, PA) containing 20 ml of MYPS (1% malt extract, 0.1% yeast extract, 0.1% tryptone and 0.1% dextrose) liquid medium and incubated for 5 days at room temperature in the dark. Preventing exposure to light suppressed melanization of the culture, resulting in a higher yield of DNA. After 5 days, cultured mycelia were collected by vacuum filtration using Whatman™ filter paper (9.0 cm in diameter) until slightly dry, washed with sterilized water, and transferred to 50 ml Falcon™ tubes. The mycelia were lyophilized for 24 h and stored at −20°C or −80°C. *Agrobacterium tumefaciens* AGL-1, from a 15% glycerol stock stored at −80°C, was cultured on AMM at 28°C for 2 days (Chen et al., 2000). AMM was supplemented with ampicillin (200 µg ml^{-1}) for selection of AGL-1 and with kanamycin (100 µg ml^{-1}) to maintain binary vectors (Tables 2 and 3).

Table 1. Fungal strains used in this research

Strains	Genetic traits[a]	Relevant characteristics
Phoma medicaginis		
P2	Wild-Type	
P2-65	Hyg^R, GFP, $G418^R$, *cid13*::*TDNA*	Fewer, smaller pycnidia and aerial hyphae
P2K10B	Hyg^R, *cid13*::*TDNA*	Partially
P265C8	Ntc^R	Partially recovered phenotype of P2

[a] Hyg^R = hygromycin B resistance (*hph*); GFP = green fluorescent protein; $G418^R$ = G418 resistance (*nptII*); Ntc^R = nourseothricin resistance (*nat1*)

Table 2. Bacterial strains used in this research.

Species	Strain	Binary Vector	Bacterial Selection[a]	Fungal Selection[b]	Visual Marker
Agrobacterium tumefaciens	AGL-1	N/A	amp	N/A	N/A
A. tumefaciens	AGL-1	pBHt2.tdTom	amp, kan	Hyg^R	tdTom
A. tumefaciens	AGL-1	pCB403.2	amp, kan	Hyg^R	N/A
A. tumefaciens	AGL-1	pCBCID13-hph	amp, kan	Hyg^R	N/A
A. tumefaciens	AGL-1	pCAM-Nat1	amp, kan	Ntc^R	N/A
A. tumefaciens	AGL-1	pCAM-Nat1::*PmCID13*	amp, kan	Ntc^R	N/A

[a] amp = ampicillin or carbenicillin (*bla*); kan = kanamycin (*nptIII*)

[b] abbreviations as in Table 1

Plasmids, primers and conventional polymerase chain reaction (PCR):

Table 3. Plasmids used in this research

ATMT plasmids	Genetic traits[a]	Relevant Characteristics
pPTGFPH	Kan^R, Hyg^R, GFP, $G418^R$	T-DNA tagging
pCB403.2	Kan^R, $G418^R$	Gene deletion vector
pCB403.2 (*Δcid13*::*hph*)	Kan^R, $G418^R$, Δ*cid13*, Hyg^R	Gene deletion of *CID13*, G418R counter
pCAM-Nat1	Kan^R, Ntc^R	Complementation vector
pCAM-Nat1(*PmCID13*)	Kan^R, Ntc^R, *CID13*	Complementation of *cid13*

[a] abbreviations as in Tables 1 and 2

Genomic DNA isolation: In order to isolate high quality and quantity of genomic DNA for Southern blot hybridization, wild-type strain P2 and the three transformants, original mutant P2-65, knockout mutant P2K10B and complemented mutant P265C8, were cultured and lyophilized as described above. Genomic DNA was isolated according to the following modified method (Möller et al., 1992):

1. 40 mg lyophilized mycelia was homogenized by bead beating with one 6 mm, three 3 mm beads and ~200 µl 0.5 mm zirconium beads. To this mycelial powder, 500 µl TES buffer (100 mM Tris, pH 8.0, 10 mM EDTA and 2% SDS) with 1.0% PVP, 1.0% PVPP, 0.1% β-mercaptoethanol and 100 ug Proteinase K, were added

and incubated 1 h at 60℃ shaking at 350 rpm in a thermomixer (Eppendorf, Hauppauge, NY).

2. The salt concentration of the homogenate was adjusted to 1.4 M with 5 M NaCl, 0.1 volume 10% cetyltrimethyl ammonium bromide (CTAB) added, and incubated for 10 min at 65℃.
3. One volume SEVAG (chloroform: isomylalchohol (24:1)) was added and the homogenate mixed gently and incubated for 30 min at 0℃, and then, centrifuged for 10 min at 4℃, rpm max. Transfer upper phase (aqueous supernatant) to a new 1.5 ml tube.
4. Repeat step 3 with the supernatant.
5. To the supernatant, add 225 µl 5 M ammonium acetate and mix gently; place on ice for over 1 h; centrifuge at 4℃, rpm max. Transfer supernatant to a new 1.5 ml tube.
6. Add 2 ul of RNase I (200 mg ml^{-1}) and incubate 30 min at 37℃.
7. Add 0.55 vol isopropanol to precipitate DNA and centrifuge for 5 min, rpm max
8. Aspirate off supernatant and wash pellet twice with cold 70% ethanol and centrifuge 5 min at rpm max
9. Air dry pellet and dissolve in 50 µl Tris-EDTA (TE) buffer

This modification such as twice of SEVAG extraction allowed high yields of DNA to be purified effectively by removing phenolics and polysaccharides from genomic DNA. Commercial DNA isolate kits did not isolate sufficient yields of genomic DNA, even though purity was high.

RNA isolation: Mycelia of P2, P2-65, P2K10B, and P265C8 were cultured as before for DNA in MYPS in the tissue culture flasks for 14 days. After 14 days, the cultures were harvested by vacuum filtration, washed several times with sterilized water, and then frozen with liquid nitrogen. RNA was isolated using the following protocol:

1. Add 1 ml TRIzol to 50 mg frozen mycelium and pycnidia and beadbeat as before, for 30 sec, twice.
2. Incubate shaking for 5 min at room temperature
3. Add 0.2 ml chloroform and beadbeat at max speed for 15 sec.
4. Centrifuge at 12,000 *g* for 10 min at 4℃ and transfer the supernatant to a new1.5 ml tube.
5. Add 0.5 ml isopropanol and mix well.
6. Gently shake for 10 min at RT.
7. Centrifuge 12,000 *g* for 10 min at 4℃ and discard supernatant.
8. Wash pellet using 75% ethanol, vortex, and centrifuge at 7,500 *g* for 5 min at 4℃
9. Air dry pellet at RT.
10. Dissolve pellet in 40 µl diethylpyrocarbonate (DEPC)-treated water and 10 µl 5× RNAsecure (Ambion, NY, USA) and incubate at 60℃ for 10min
11. RNA samples were purified using RNA cleanup Rneasy mini kit (Qiagen) according to the manufacturer's instructions.

The final concentrations of RNA solution were measured by NanoDrop 1000 (Thermo Fisher NanoDrop, Wilmington, DE) and 5 µg aliquots of isolated RNA stored at −80°C. Isolated RNA was used to efficiently synthesize cDNA.

First strand cDNA synthesis: cDNA was synthesized from total RNA isolated from mycelia and pycnidia cultured in MYPS. The cDNA synthesis reactions were set up in 20 µl volumes containing 5 µg total RNA, 1ul of 50 µM oligo$(dT)_{20}$, 10 mM dNTP mix and DEPC-treated water to 20 µl and incubated at 65℃ for 5 min. Afterwards, the following components were added to each reaction: 2 µl 10X RT buffer, 4µl 25mM $MgCl_2$, 2 µl 0.1 M DTT, 1 µl RNaseOUT and 1 µl SuperScript III reverse transcriptase (200 U; Life Technologies, Grand Island, NY). The cDNA synthesis reaction was carried out on a PTC-200 DNA engine thermal cycler (MJ research, Waslthan, MA) using the following program: 50 min at 50℃, 5 min at 85℃, and, after adding 1 ul of RNase H, 20 min at 37°C. All cDNA was stored frozen at −80°C.

Construction of knockout and complementation vectors: To construct the *Pmcid13* gene-replacement vector, the *Pmcid13* gene was amplified with KOD DNA polymerase (Merck Millipore, Billerica, MA) and primers containing 5' *Kpn*I and *Xba*I restriction enzyme sites. The *Pmcid13* fragment containing *Kpn*I and *Xba*I sites was cloned into *Kpn*I and *Xba*I sites of pGEM-4z cloning vector (Promega, Madison, WI). This process resulted in a vector, pGEM4_cid13. A 2593 bp OHT cassette containing the selectable marker hygromycin B resistance (*hph*) under control of the *Aspergillus nidulans oli*C promoter and *Botrytis cinerea tuba* terminator was amplified (as above) from the pOHT vector by using primers engineered with 5' *Apa*I and *Not*I sites. The pGEM4_cid13 vector and the amplified OHT cassette were digested with *Apa*I and *Not*I and the larger backbone pGEM4_cid13 fragment ligated with the digested OHT, producing

pGEM4_cid13_hph. A 5.2 kb fragment containing 5' *cid13* flanking DNA::OHT cassette::3' *cid13* flanking DNA released from pGEM4_cid13_hph by digesting with *Sbf*I and *Sac* I and cloned into the same sites in the binary vector pCB403.2, creating the pCBcid13_hph knockout vector. The complementation vector pCAM-Nat1 (described in previous chapter) and the Pmcid13 amplicon (described above) were digested with *Kpn*I and *Xba*I and ligated to create the Pmcid13 complementation vector pCAM-Nat1_cid13. Both the knockout and complementation vectors were transformed into and maintained in *E. coli* DH5-α and *A. tumefaciens* AGL-1 as described in the previous chapter. ATMT was performed, as described in the previous chapter, with conidia of the wild type P2 with the knockout vector and conidia of the mutant P265 with the complementation vector.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR): The cDNAs from each strain, P2, P2-65, P2K10B and P265C8, were used as templates for PCR. The primers, 3'p265 exons and p265.gap3 were used for RT-PCR to amplify the intron between exon 1, exon 2 and exon 3 of Pmcid13, producing a larger band from genomic DNA (315 bp) than from cDNA (208 bp), and thus indicating if contaminating genomic DNA was present. Twenty µl PCR reactions contained 12.5 ul of 2X GoTaq Green Master Mix (Promega, Madison, WI), 100 nM each primer, 10-20 ng cDNA and ddH_2O to 20 ul. The PCR program was as follows: 95℃ for 5 min, followed by 30 cycles of 94℃ for 30 sec, each primer pair's (3'p265exons and Fp265gap3) average annealing temperature for 30 sec, and 72℃ for 1 min/Kbp. All PCR reaction were carried out with

'no DNA' negative controls and genomic DNA of each strain as positive controls for each primer set using a PTC-200 DNA engine thermal cycler.

Table 4. Primers used in this study

Name	Sequence (5' to 3')	Tm	Description
3'p265exons	CCTGGGAATCTATCCAAAGT	54.5	Amplify exon 1, 2 and 3
Fp265gap3	GCTACGTTGTTCACGTTCAT	57.2	Amplify exon 1, 2 and 3
5'RTP265-2	GCAACCTCCACTTAGATCACTA	58.2	Confirm complementation vector with Nat-R
Hyg-R	GCCGATGCAAAGTGCCGATAAACA	64.5	Confirm KO vector
M13 reverse (27)	CAGGAAACAGCTATGAC	50.3	Confirm KO vector
Nat-R	ATCGCCGGTGCGTTGACGTT	62.6	Confirm complementation vector with 5'RTP265-2
BstXI- trpC-F	CGCCAACATGGTGGACAGAAGATGA	64.2	Confirm complementation strains with Nat1-R
KpnI_pmcid13	AACAGGTACCGCGATGCTAGGCTGG	65.5	Amplify *Pmcid13* in P2 for pCAM-Nat1-cid13
XbaI_pmcid13	AGCTTCTAGACTTCGGACGTGATTAT	62.0	Amplify *Pmcid13* in

			P2 for pCAM-Nat1-cid13
ApaI-oliC	GGAAGGGCCCCTGCAGCTGTGGAGC	73.4	Amplify oliC-hph for KO vector
NotI_hph	CGATGCGGCCGCAAGCTTGATATCTG	64.3	Amplify oliC-hph for KO vector
P265RBconf2	CATGGTTCTGGTCAGGAGTA	53.7	Amplify 5' exon4
P265GW2	ATC GCG CCA ACA GCA TAG TCT TGT AGT	61.8	Amplify 5' exon4
5'P265exons2	AGATTGAATCCACCTCTTCC	51.7	Make probe1 and amplify exon3
FP265_gap3	GCTACGTTGTTCACGTTCAT	53.3	Make probe1 and amplify exon3
FP265confGWLB	CTGTGCACCACTGCTATTCA	53.1	Make probe2
P265GWLB	AGACATCAAGGTGCCAATAACAACAC	57.7	Make probe2
F-P265GAP2	CGTCGGCTATTGGTGTCTAT	54.2	Amplify 3' exon4
P265LBconf3	CGTTTGTTGCTTTGCTATTC	50.5	Amplify 3' exon4

Preparation of Labelled Probes for Southern Hybridization : Two probes were amplified from P2 genomic DNA and one probe was amplified from the knockout vector. These three gene-specific probes were amplified from the 3' flanking DNA of *cid13* gene (644 bp), a portion of the Pmcid13 region deleted by knockout vector (729 bp) and the *hph* gene (684 bp) using primer sets: F-p265GAP1 and 5'p265exons, p265GWLB&F

and p265confGWLB, and Hyg-F and Hyg-R, respectively, using GoTaq Green Master Mix (Promega, Madison, WI). The PCR programs for the three probes were as follows: an initial denaturation at 95℃ for 5 min; 30 cycles of 95℃ for 30s, 55℃ or 60.3℃ for 30s and 72℃ for 1min; followed by an additional 10 min extension at 72 ℃. These amplicons were purified using a PureLink PCR purification kit (Invitrogen, Carlsbad, CA) and labelled using the AlkPhos Direct Labeling and Detection System (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. Blotted dilution series of each probe and genomic DNA were used to test labeled probes' limits of detection. Five ug genomic DNA from each strain and each nonlabeled probe were spotted in a $\log_{10}$ serial dilution (10^{-1} to 10^{-8}) on a positively charged nylon membrane (Millipore, Billerica, CA) and hybridized with labeled probe after UV-cross liked according to the manufacturer's instruction (GE Healthcare, Piscataway, NJ). Briefly, spotted membrane were pre-hybridized with hybridization buffer from the kit for 20 min at 55℃, then labeled probes (10ng/ul) were added into hybridization bottle, and incubated overnight at 55℃. The next day, the membrane was washed with primary and secondary washing buffer twice for 15 min at 55ºC according to manufacturer's instruction.

Southern Blot: Southern blots were conducted using conventional molecular methods (Sambrook et al., 1989). Once genomic DNA of each strain was isolated (see above), 20 to 40 ug was digested with *Bam*HI and *Acu*I and *Stu*I at 37℃ overnight and separated on a 0.8% Tris-acetate-EDTA (TAE) agarose gel at 50 V for 2 h. After electrophoresis, the gel was post-stained with ethidium bromide. Separated DNA was transferred to a

positively charged nylon membrane by upward capillary transfer using 10X saline-sodium citrate (SSC). The blot was fixed after air drying by UV crosslinking. The denatured purified probes were labelled with alkaline phosphatase using the Alkaphos Direct kit (Amersham Biosciences, Pittsburgh, PA) and hybridized with the blot overnight at 55℃ in a hybridization oven. Labeled probes were stored in 50% (v/v) glycerol at -20°C according to manufacturer's instructions.

Signal detection using CDP-Star: The signal of hybridized probe on the blot was generated by chemiluminescence using CDP-Star (Tropix, Inc. Bedford, MA) as a substrate. After incubating hybridized blots with CDP-Star, blots were exposed to chemilumenescence grade autoradiography film CL-Xposure Film (Thermo Scientific, Waltham, MA) inside a photography cassette for 5 h. The exposure time was increased up to overnight depending on the strength of signal.

Hydroxyurea (HU): For the HU sensitivity test, agar plugs of P2, P2-65 and P2K10B were inoculated onto triplicate YPS plates containing different concentration of HU (Sigma; 0 mM, 1mM, 2mM and 5 mM). These were incubated at 18-20℃ under lights and colony diameters measured daily.

DNA synthesis inhibitors: Inhibitors of DNA synthesis used in this study are described in Table 5.

Table 5. Inhibitors of DNA synthesis used in this study (Cozzarelli, 1977)

Inhibitor	Interaction with DNA	Sensitive organisms	concentration for test
Chloroquine	Int[a]	Eukaryotes	0 to 500 ug/ml
Cytarabine	Yes	Eukaryotes and virus	0 to 500 ug/ml
Gallium III nitrate	Yes	Eukaryotes, bacteria	0 to 500 ug/ml
5-Fluorouracil	No	Eukaryotes and bacteria	0 to 5 ug/ml

[a]int: intercalation

Agar plugs of strains P2, P2-65, P2K10B and P265C8 were inoculated onto triplicate YPS plates containing various concentrations of DNA synthesis inhibitors. These cultures were incubated at 18-20℃ under lights and colony diameters measured daily.

Virulence Assays of wild-type and transformants: Conidia of each strain, P2, P2-65, P2K10B and P265C8, were collected from 14 day old MYPS agar cultures. The three of 3 mm agar culture plug from 14 days MYPS were beadbeated (BioSpec Products, Inc., Bartlesville, OK) using one 6 mm and three 3 mm glass beads in 1 ml sterilized water. Conidial suspension from bead beat mycelial fragments were quantified using a hemacytometer and the concentration of the conidia adjusted to 1×10^6 spores/ml with sterile water and 50 ppm Tween 20. The spring black stem-susceptible alfalfa (*M. sativa*) cultivar, Vernal, was planted into multi cell plug trays and placed in a growth chamber at 25℃ with a 12 hour light cycle. Before planting, seeds were surface sterilized using a 1%

bleach (~0.05% NaOCl), 10% ethanol and washed 3 times with distilled water. The resulting 4 week old plants were inoculated with each strain as 15 ml spore suspensions using sprayer until runoff. For the mock inoculations, sterilized water with 50 ppm Tween 20 was sprayed to plants. After 10 days post-inoculation (dpi), the plants moved to a moist chamber under darkness at 20℃. The symptoms on the leaves and petioles were first recorded at 20, 27 and 34 dpi with following rating scale.

Table 6. Rating scale for spring black stem and leaf spot of alfalfa (http://www.naaic.org/stdtests/spring.pdf).

Rating scale	Symptom
1-resistant	Healthy, symptom-free top growth
2-resistant	Small (<2 mm), browm or black lesions or flecks; no defoliation
3-susceptible	Large (2 to 3 mm), disease lesions; lesions may be on leaves and petioles; usually no chlorosis or defoliation
4-susceptible	Large (> 3mm), fruiting, dead leaves or defoilation
5-susceptible	Lesions > 3 mm, fruiting, dead leaves or defoliation

Statisical Analyses: Multiple comparisons between strains means were conducted by the least significant difference (LSD) method at a significance level of 0.05.

Results

Morphological characteristics of wild-type and transformants

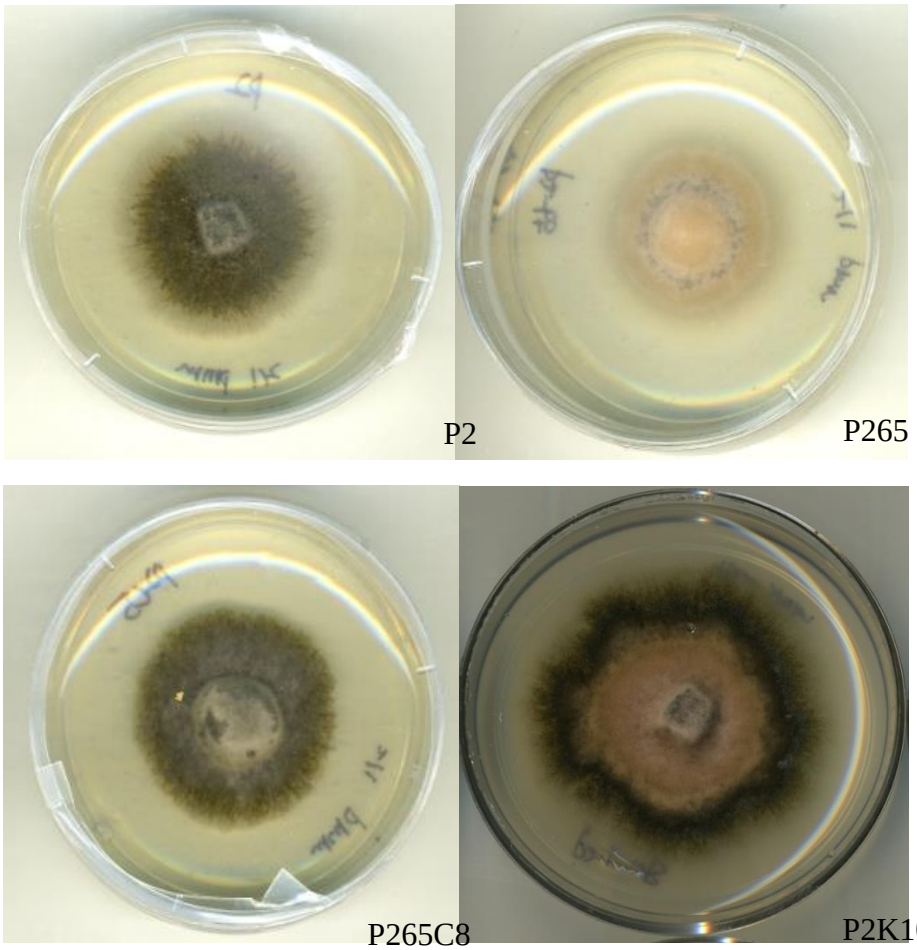


Fig 5. Cultural phenotypes of wild type (P2), Pm*cid13::TDNA* (P2-65), gene deletion strain Δ*cid13* (P2K10B) and complement strain *cid13*/CID13 (P265C8) on PMM (7 dpi)

The wild type strain, P2 initially produced white mycelium that later became olive-green in color with white margins and T-DNA tagged mutant, P2-65 showed more aerial hyphae and less pycnidia compared to P2 strain on PMM (Fig 1). ATMT was used to

generate Pm*cid13* knockout and complementation transformants, P2K10B and P265C8, respectively. The P2K10B strain showed cultural characteristics, aerial hyphae and fewer, smaller pycnidia, similar to those of the original T-DNA disrupted P2-65 (Fig 2). The complementation mutant, P265C8 recovered some of the morphological characteristics of the wild type strain P2 on PMM, including increased pycnidia and less aerial, more melanized hyphae. The pycnidia of each strain were observed under microscope. Pycnidia were not formed at margin of colonies of mutant Pm*cid13* strains, P2-65 and P2K10B, while pycnidia were seen near the margins of the wild type and P265C8 strains in 5 day old culture on YPS (Fig 2).

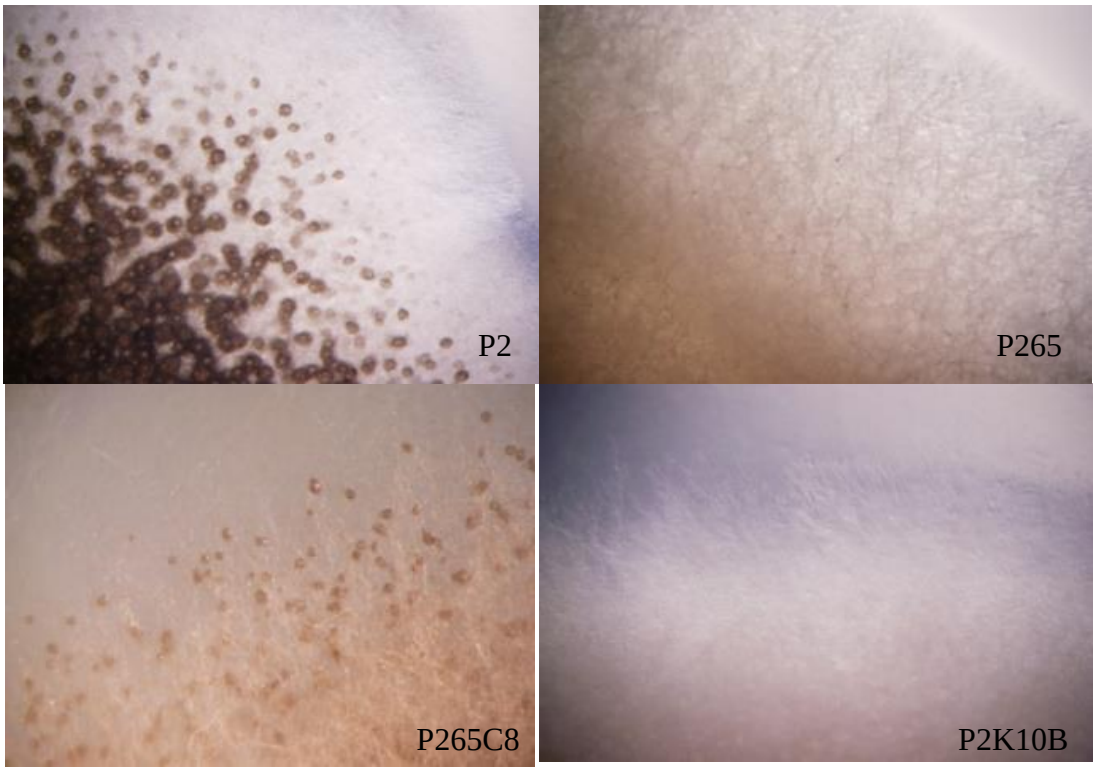


Fig 6. Pycnidia of wild type (P2), Pm*cid13::TDNA* (P2-65), gene deletion strain ΔPm*cid13* (P2K10B) and complementation strain Pm*cid13*/PmCID13 (P265C8) on YPS (7 dpi) 100x

Table 7. Size of pycnidia of wild type and mutant transformants formed on filter papers

Strain	P2	P265	P2K10B	P265C8
Size of pycnidia (μm)[a]	255.94±56.78	189.64±78.32	147.22±73.84	164.40±45.33

[a] mean diameter of 20 pycnidia ± the standard deviation

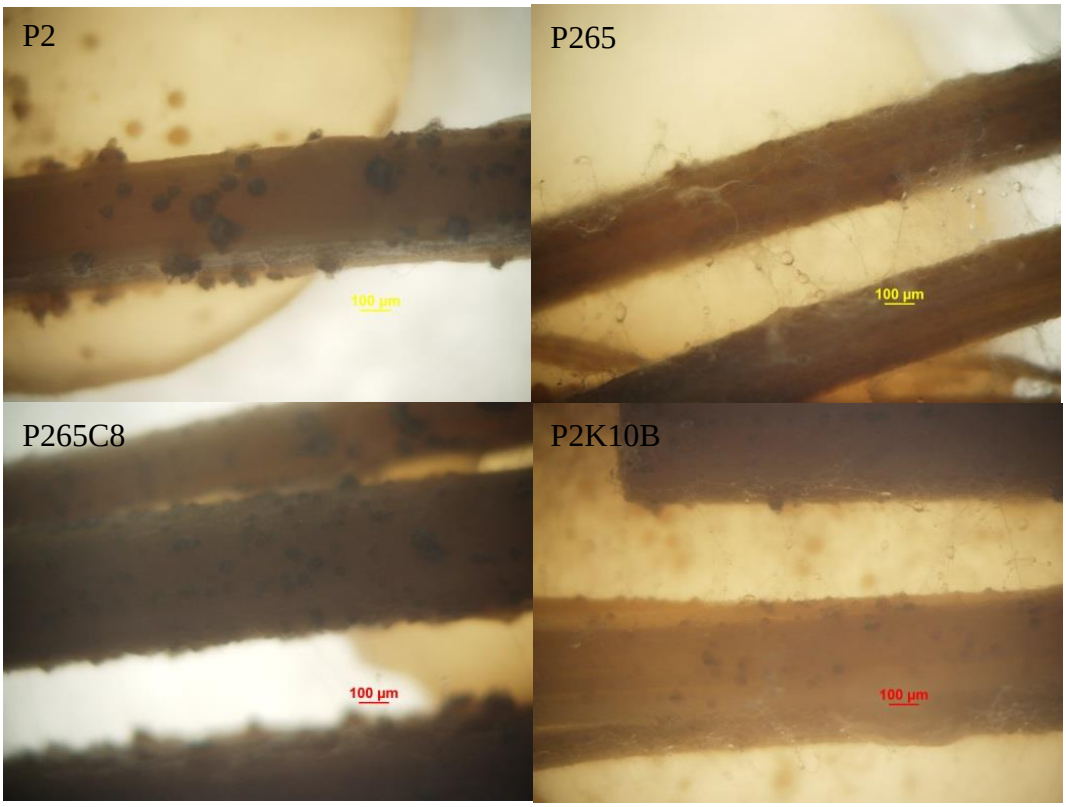


Fig 7. Pycnidia of wild type (P2), Pm*cid13::TDNA* (P2-65), gene deletion strain ΔPm*cid13* (P2K10B) and complement strain *cid13*/CID13 (P265C8) on alfalfa stems (7 dpi)

Each strain was inoculated onto sterilized alfalfa stems to investigate the formation of pycnidia. Wild type strain P2 formed pycnidia on the stems of alfalfa, whereas pycnidia of *cid13*::*TDNA* P2-65 did not (Fig 4). The Pm*cid13* deletion mutant, P2K10B developed smaller pycnidia compared to P2 and CID13 complemented mutant, P265C8 produced more pycnidia, although the size of pycnidia did not recover wild type diameters (Table 6). Pycnidial formation on the filter papers using alfalfa stems. However, P265C8 did appear to recover some of P2's ability to form pycnidia on the underlying filter paper, while P265 and P2K10B form pycnidia only adjacent to the agar plugs (Fig 4).

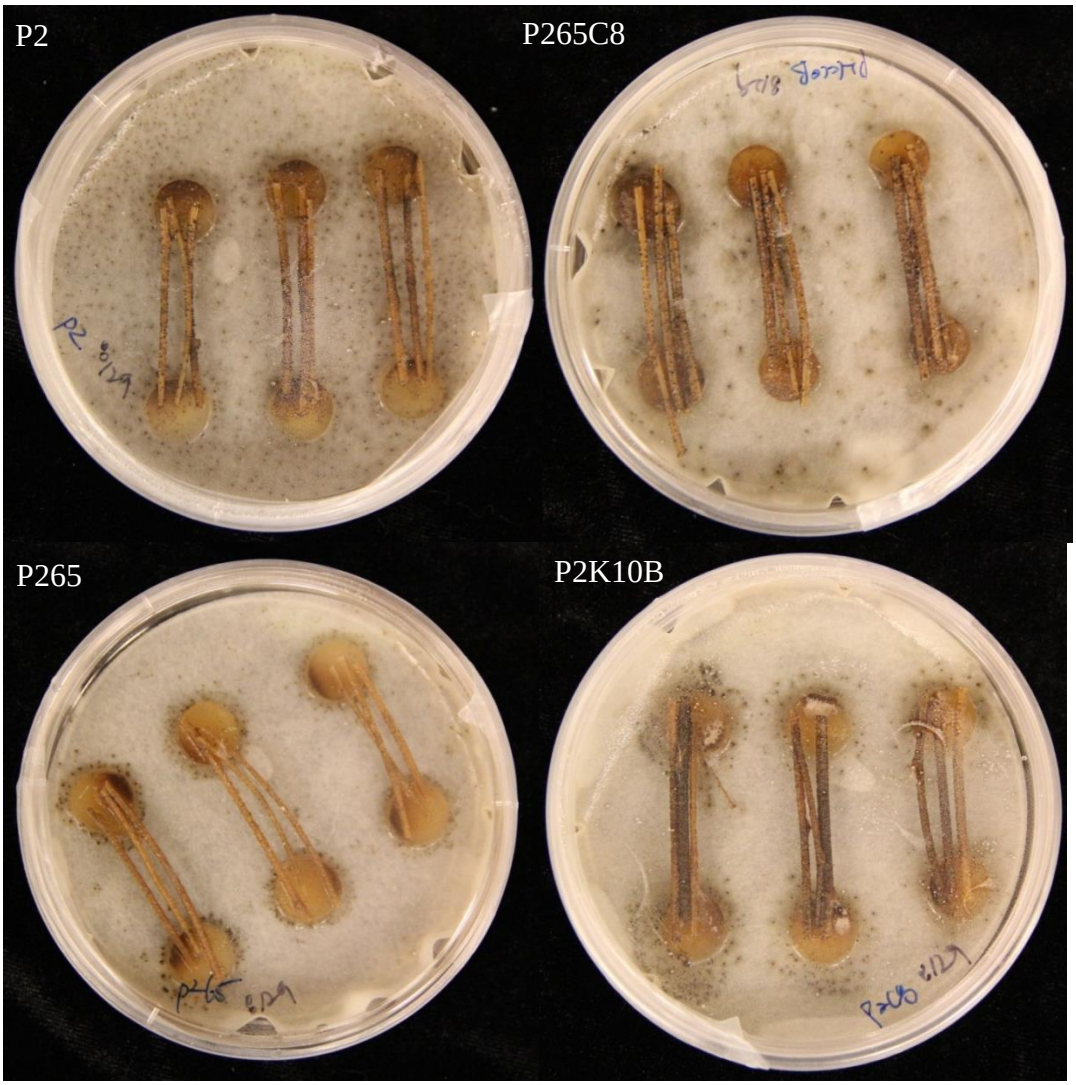


Fig 8. Pycnidia of wild type (P2), Pm*cid13::TDNA* (P2-65), gene deletion strain ΔPm*cid13* (P2K10B) and complement strain *cid13*/PmCID13 (P265C8) on alfalfa stems inoculated by placing on agar plugs of each strain (14 dpi).

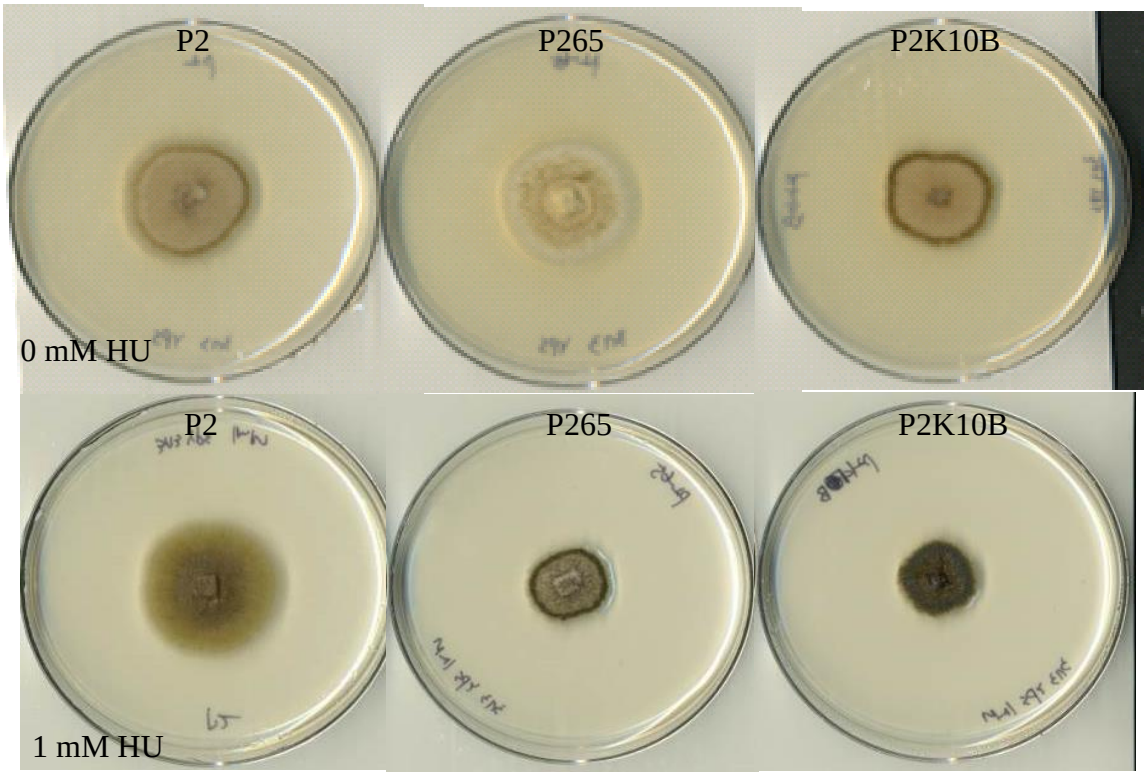


Fig 9. Hydroxyurea (HU), a ribonucleotide reductase (RNR) inhibitor, inhibits growth of Pm*cid13* mutants P265 and P2K10B more than wild type P2 on YPS (6 dpi).

The wild type (P2) was viable and grew to similar diameters on YPS containing 0 and 1 mM HU though with less aerial mycelium and more melanization with HU (Fig 5). On the other hand, mutant Pm*cid13::TDNA* (P2-65) and gene deletion strain ΔPm*cid13* (P2K10B) were sensitive to 1 mM HU, showing both inhibited colony diameters and more melanization.

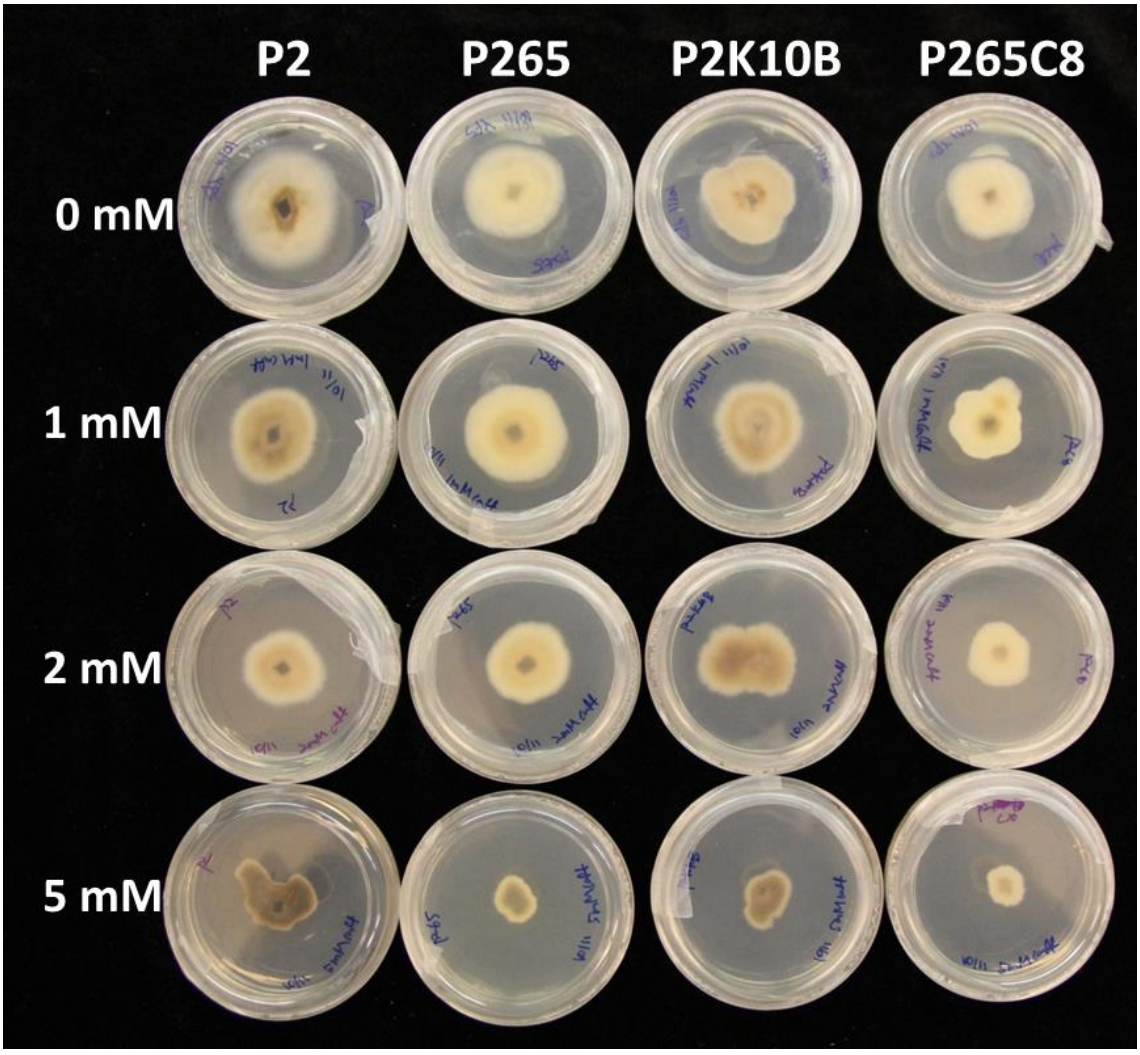


Fig 10. YPS plates containing various concentrations of caffeine inoculated with conidia of wild type (P2), Pm*cid13::TDNA* (P2-65), gene deletion strain ΔPm*cid13* (P2K10B) and complement strain Pm*cid13*/PmCID13 (P265C8) (7 dpi).

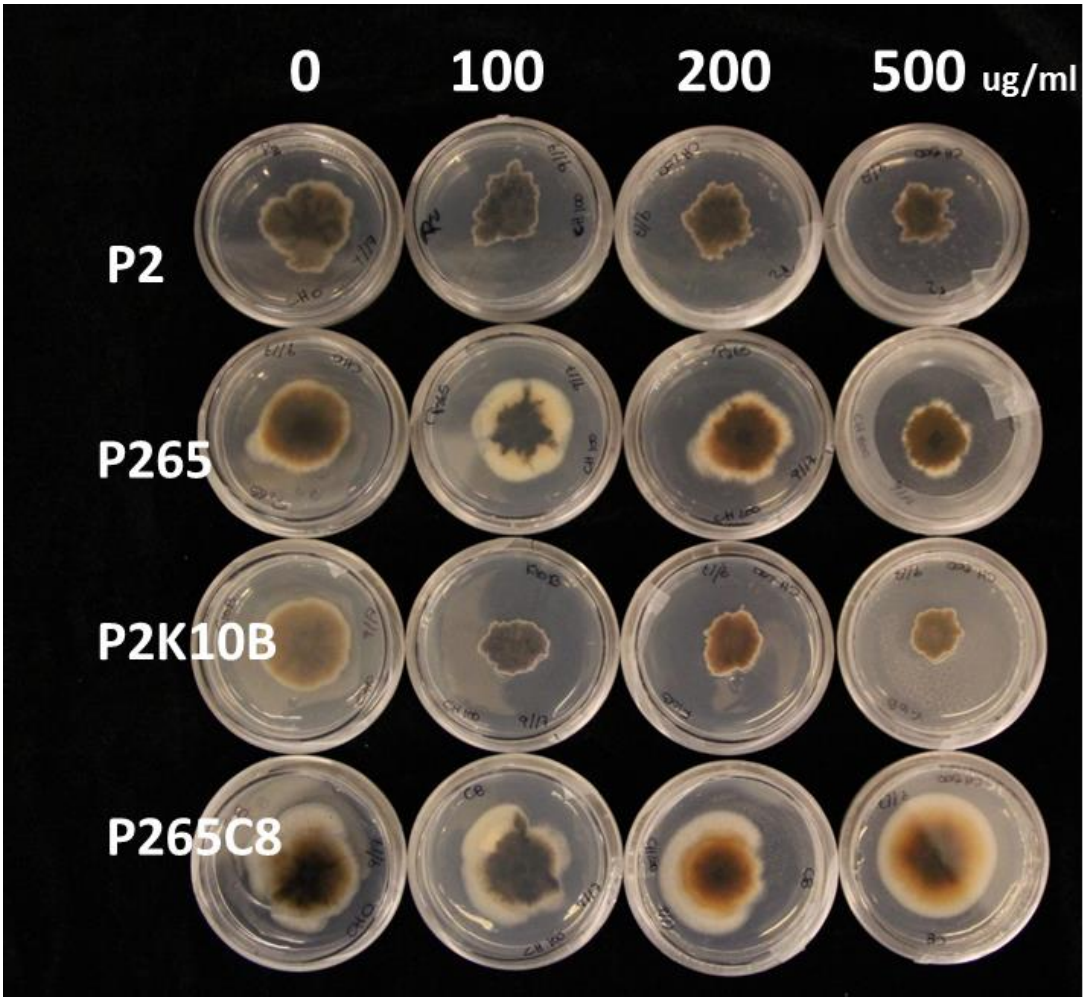


Fig 11. YPS plates containing various concentrations of chloroquine inoculated with conidia of wild type (P2), Pm*cid13::TDNA* (P2-65), gene deletion strain ΔPm*cid13* (P2K10B) and complement strain Pm*cid13*/PmCID13 (P265C8) (7 dpi).

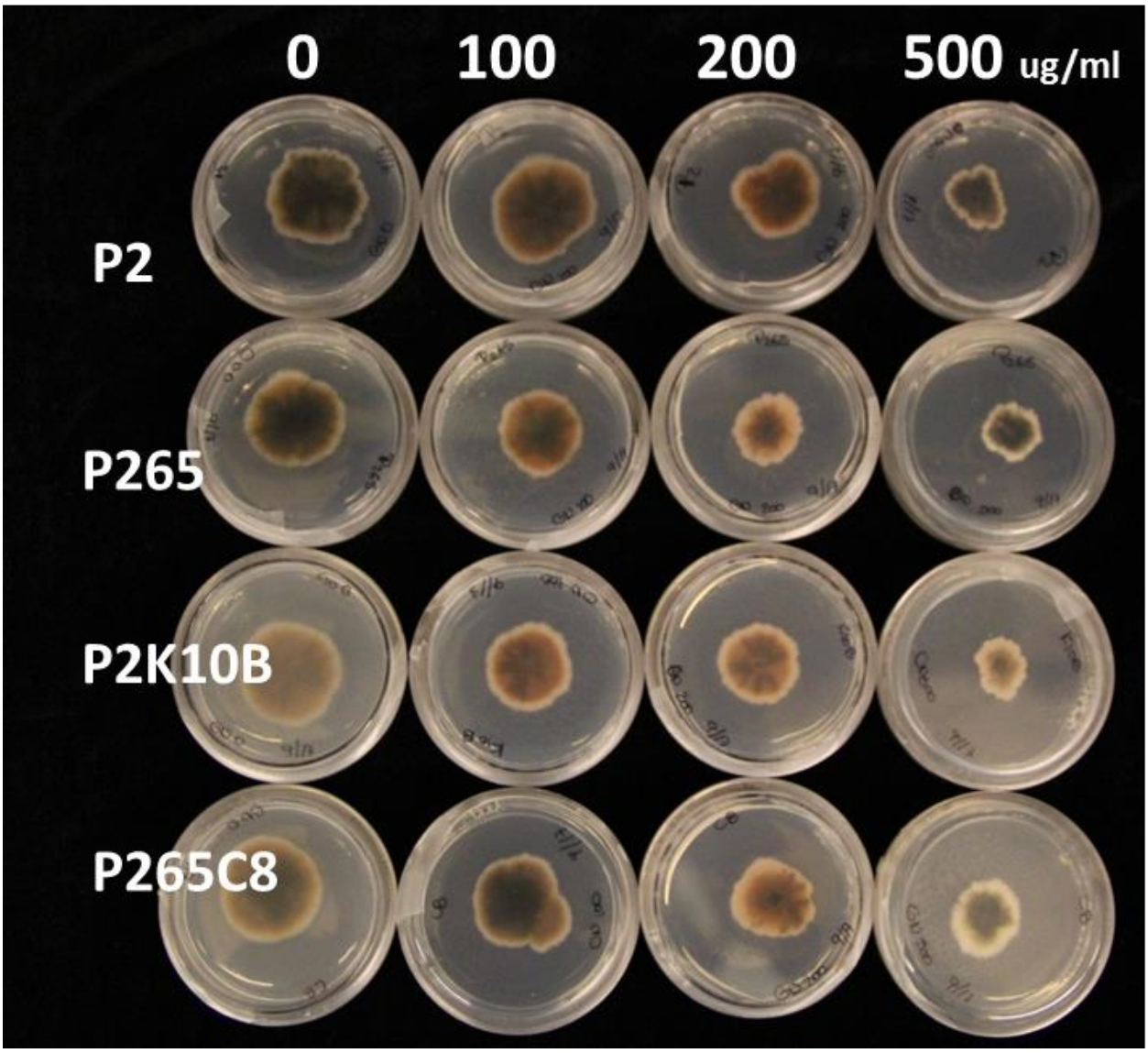


Fig 12. YPS plates containing various concentrations of Gallium III nitrate inoculated with conidia of wild type (P2), Pm*cid13::TDNA* (P2-65), gene deletion strain ΔPm*cid13* (P2K10B) and complement strain Pm*cid13*/PmCID13 (P265C8) (7 dpi).

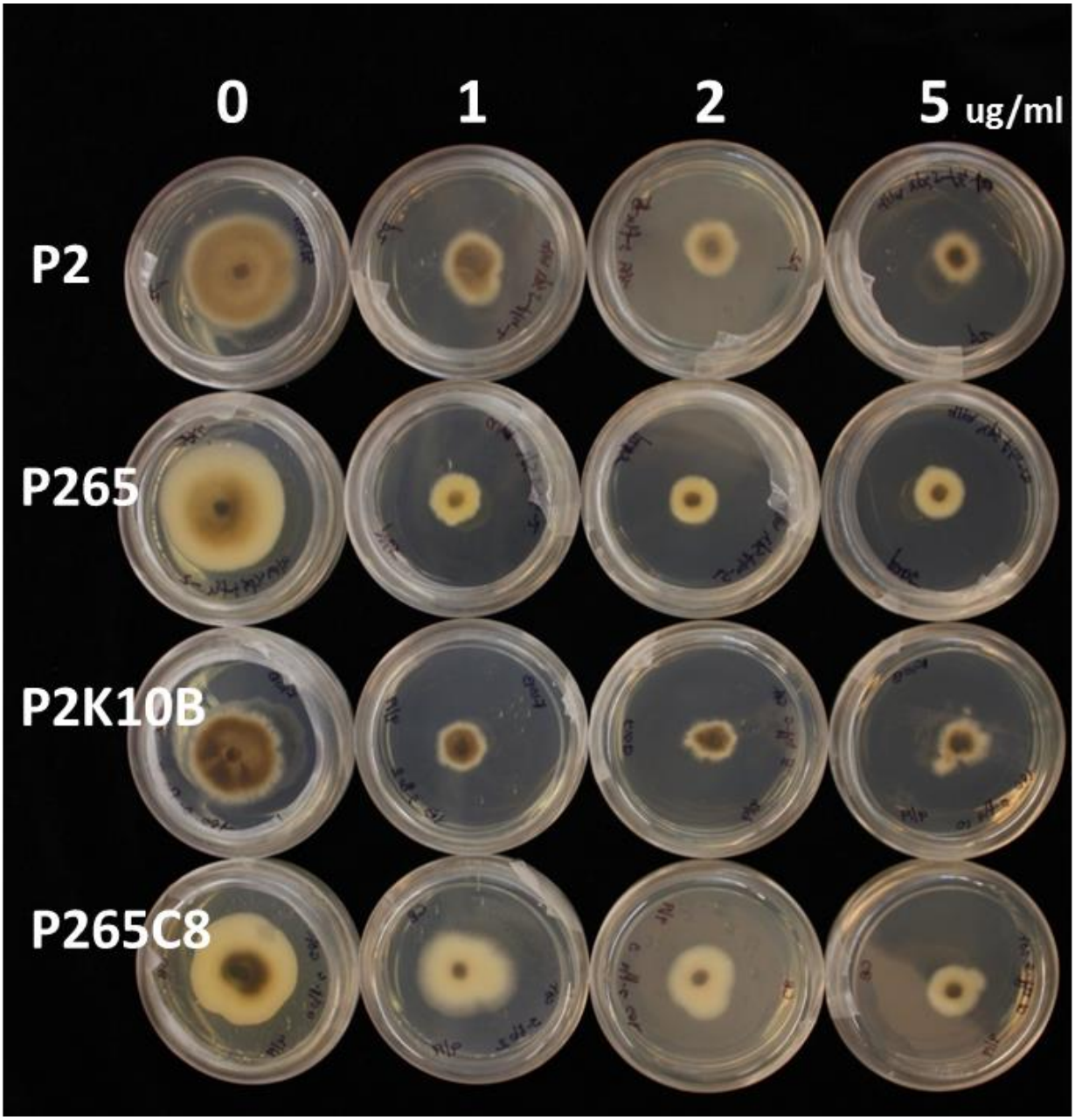


Fig 13. YPS plates containing various concentrations of 5-fluorouracil inoculated with conidia of wild type (P2), Pm*cid13::TDNA* (P2-65), gene deletion strain ΔPm*cid13* (P2K10B) and complement strain ΔPm*cid13*/PmCID13 (P265C8) (7 dpi).

The sensitive test of DNA synthesis inhibitors showed that only P2K10B strain was more sensitive to chloroquine and 5-fluoreuracil.

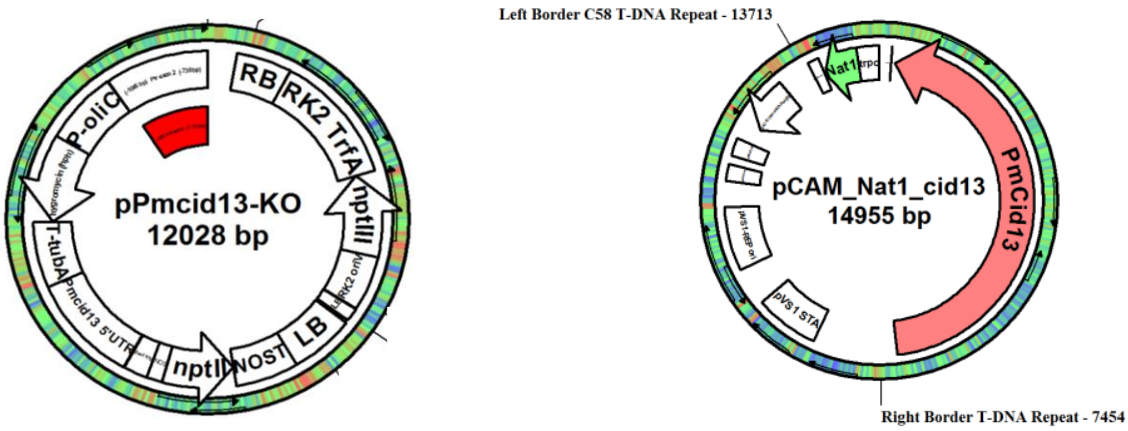


Fig 14. Pmcid13 knockout and complementation ATMT vectors, pPmcid13-KO and pCAM_Nat1_cid13.

Construction of Pm*CID13* knockout and complementation vectors

In order to identify the role of Pm*CID13* in *P. medicaginis*, the Pmcid13 knockout vector, pPmcid13-KO was constructed with the backbone of pCB403.2 binary vector. The Pmcid13 complementation vector, pCAM-Nat1-cid13 was constructed using the backbone vector pCAMBIA1300 (CAMBIA, Canberra, Australia).

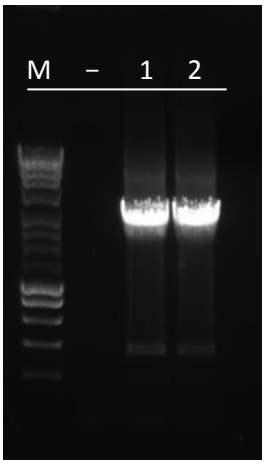


Fig 15. PCR and colony PCR of hph and 3' flanking DNA of *Pmcid13* on agarose gel confirmation of knockout vector, pPmcid13-KO. Lane 1: pPmcid13-KO, lane 2: E. coli containing pPmcid13-KO, M: 1 kb DNA ladder and −: negative control (water).

The knockout vector, pPmcid13-KO was constructed as described above in the Materials and Methods and was verified by PCR using the M13 reverse and HPH forward primers (Fig 11). The expected amplicon size is 2263 bp. Also, the OHT cassette flanked by the 5'- and 3'-DNA of Pm*CID13* was confirmed by sequencing (data not shown). After ATMT using *A. tumefaciens* AGL-1 (pPmcid13-KO), insertion of T-DNA into the genome of P2 knockout transformants (P2K7, P2K10B and P2K20) was confirmed by PCR using primers targeting the *oliC* promoter and *hph* region.

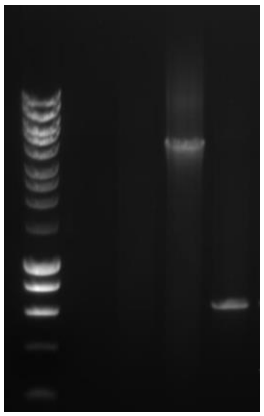


Fig 16. PCR agarose gel confirmation of complementation vector, pCAM-Nat1-cid13. Lane 1: pCAM-Nat1-cid13, lane 2: pCAM-Nat1, M: 1 kb DNA ladder marker (Apex) and and −: negative control (water)

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To complement the Pm*cid13::TDNA* in P265, the complementation vector pCAM-Nat1-cid13 containing the amplified Pm*cid13* gene from wild type strain P2 was constructed and confirmed by PCR using 5'RTP265-2 and Nat-R targeting *Pmcid13* and nat1 fragment (Fig 12) and sequencing (data not shown). The expexted amplicon size by 5'RTP265-2 and Nat-R primers is 5039 bp.

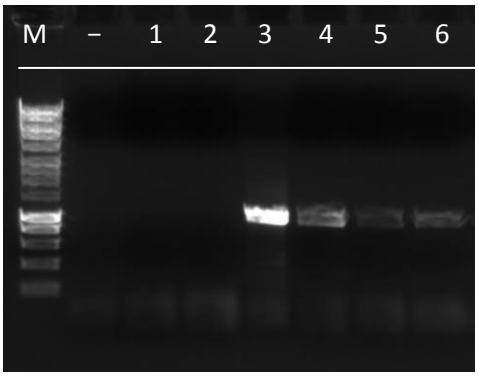

Fig 17. Confirmation of the Pm*cid13* knockout transformants by PCR amplification of the *oliC::hph* selection marker. Lane 1: P2, lane 2: P265, lane 3: positive control (pPmcid13-KO), lane 4: P2K7, lane 5: P2K10B, lane 6: P2K20, M: 1 kb DNA ladder marker (Apex) and −: negative control (water)

Analysis of Pm*CID13* knockout and complementation transformants

To assess the role of *PmCID13* in *P. medicaginis*, gene knockout and complementation transformants were generated. For gene knockout of Pm*CID13*, knockout vector pPmcid13-KO was used for transformation of *P. medicaginis* P2 to generate Pmcid13 deleted transformants (Δ*Pmcid13*) through homologous (double-crossover) recombination. In one experiment, three candidate Pm*CID13* knockout transformants, showing phenotypes most similar to P2-65 (*cid13::TDNA*), were obtained in YPS containing hygromycin (50 ug/ml). The homologous recombination at the Pm*CID13* locus was supported by sensitivity to G418 due to exclusion of the left border *nptII*, which falls outside of the Pmcid13 flanks (Fig 10), and PCR amplification of the OHT cassette in pPmcid13-KO using the *oliC* forward and *hph* reverse primers (Fig 13).

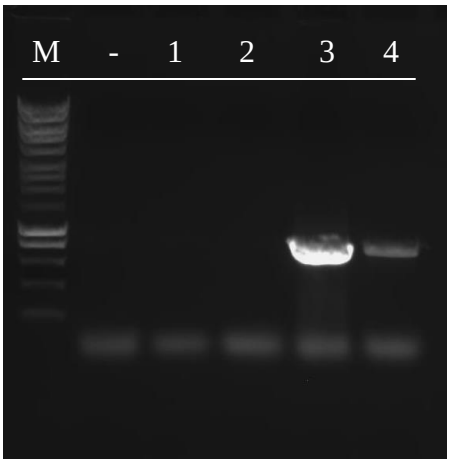

Fig 18. Confirmation of the Pm*cid13* complementation transformant by PCR. Lane 1: P2, lane 2: P265, lane 3: positive control (pCAM-Nat1-cid13), lane 4: P265C8, M: 1 kb DNA ladder (Apex) and -: negative control (water)

To confirm that Pm*CID13* is involved in altering cultural phenotypes, such as aerial hyphae and the formation of pycnidia, a wild type Pm*CID13* allele was introduced by ATMT into the disruption mutant, P2-65 (*cid13::TDNA*), using pCAM-Nat1-cid13. A total of 31 transformants were selected on YPS containing nouseothricin (50 μg/ml) and transformant selected based on its near wild type morphology. This putative complementation transformant, P265C8, was confirmed to possess the complementation vector's selective marker by PCR using the trpC forward and Nat1 reverse primers.

Analysis of expression of Pm*cid13* in wild type and transformants

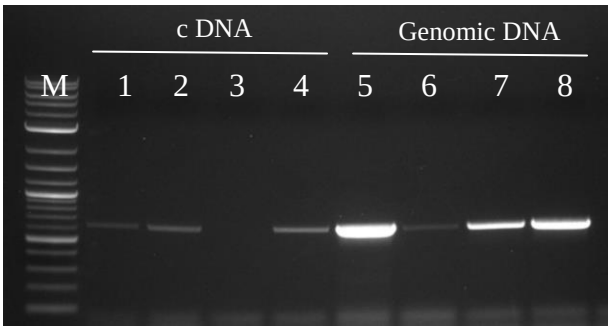

Fig 19. PCR and RT-PCR analysis of exon 1 of Pm*cid13* gene. Lanes 1, 5: P2, lanes 2, 6: P265, lanes 3, 7: P2K10B and lanes 4, 8: P265C8 and M: 1 kb DNA ladder (Apex).

To determine whether each exon of Pm*CID13* is expressed, PCR and RT-PCR using genomic DNA and cDNA as templates, respectively, were carried out. Genomic DNA and cDNA from each strain were amplified, whereas cDNA of P2K10B (Pm*CID13::hph*) was faintly amplified.

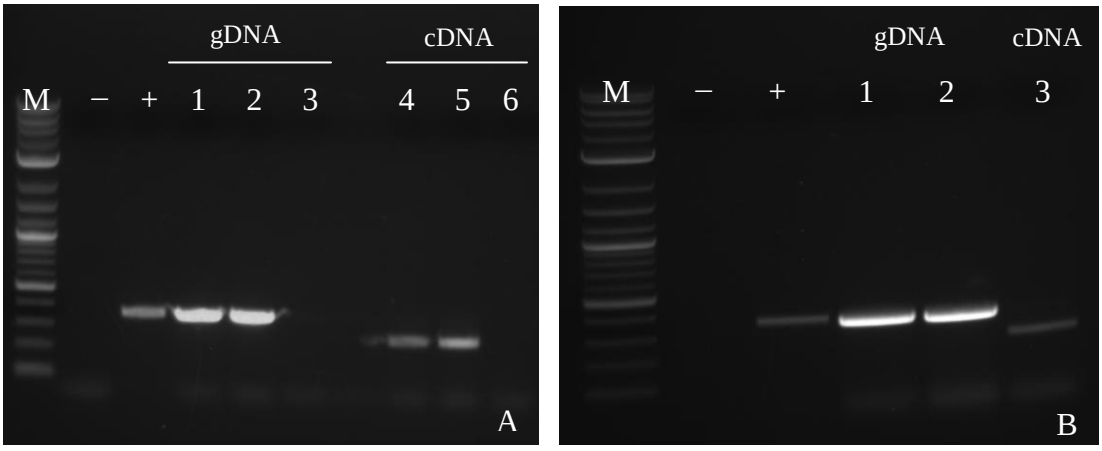

Fig 20. PCR and RT-PCR analysis of exons 1 and 2 of Pm*cid13* gene. A: lanes 1, 4: P2, lanes 2, 5: P265, lanes 3, 6: P2K10B, B: lane 1: P2, lane 2, 3: P265C8, M: 1 kb DNA ladder (Apex), −: negative control (water) and +: positive control: pCAM-Nat1-cid13

The exon 1-2 region was amplified from the genomic DNA and cDNA of P2, P2-65 (Pm*CID13::TDNA*), P2K10B (ΔPm*cid13*) and P265C8 (Pm*cid13*/Pm*CID13*) and a product size difference could be distinguished due to the splicing out of an intron apparent in the cDNA (Fig 19). The exon 1-2 region of Pm*CID13* was expressed in P2, P265, and P265C8, but not in P2K10B, indication deletion of this region was successful in the knockout transformant.

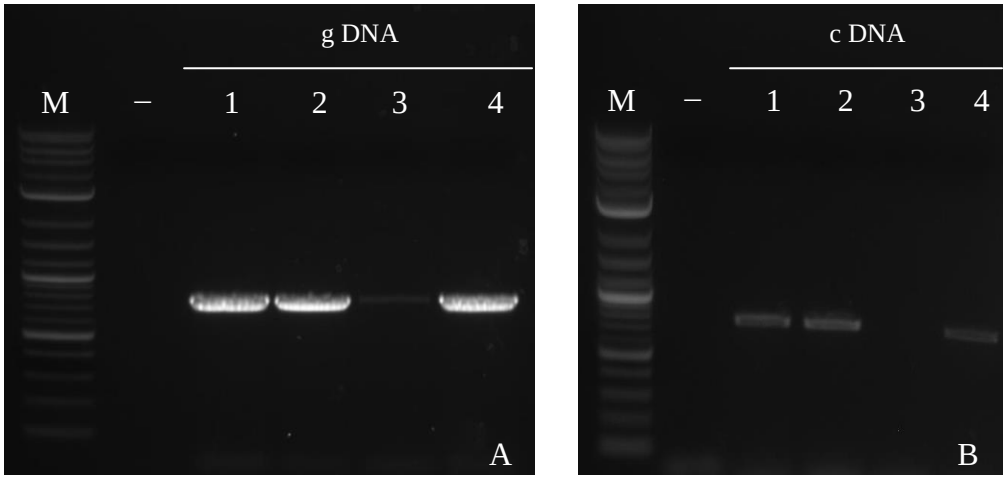

Fig 21. PCR and RT-PCR analysis of exon 3 of Pm*cid13* gene. A: lane 1: P2, lane 2: P265, lane 3: P2K10B, lane 4: P265C8 B: lane 1: P2, lane 2: P265 3: P2K10B, lane 4: P265C8, M: 1 kb DNA ladder (Apex) and −: negative control (water).

In P2K10B, exon 3 was not expressed, but was expressed in P2, P2-65, and P265C8 (Fig 16). Exon 3 was supposed to have been deleted in P2K10B, however, exon 3 was amplified weakly from the genomic DNA of P2K10B, though it was not expressed (Fig 20B, lane 3).

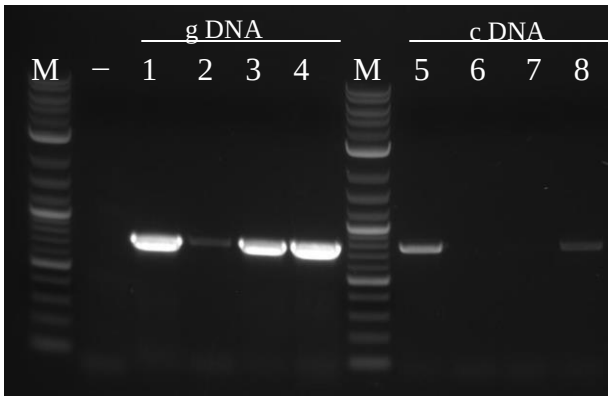

Fig 22. PCR and RT-PCR analysis of the 5’ region of exon 4 of the Pm*cid13* gene. A: lanes 1, 5: P2, lanes 2, 6: P265, lanes 3, 7: P2K10B, lanes 4, 8: P265C8, M: 1 kb DNA ladder (Apex) and −: negative control (water)

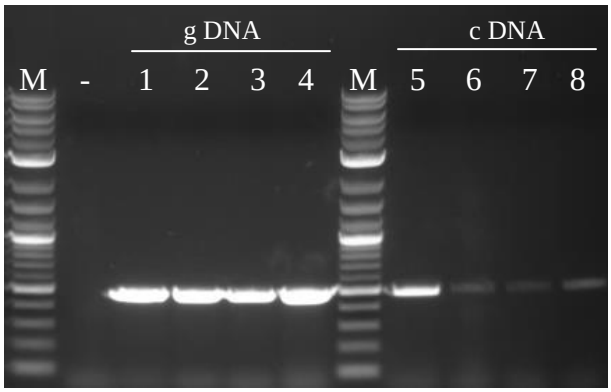

Fig 23. PCR and RT-PCR analysis of the 3’ region of exon 4 of the Pm*cid13* gene. A: lane 1, 5: P2, lane 2, 6: P265, lane 3, 7: P2K10B, lane 4, 8: P265C8, M: 1 kb DNA ladder (Apex) and −: negative control (water)

The 5’ of exon 4 was amplified from the genomic DNA of P2, P2-65 (faintly), P2K10B and P265C8 (Fig 21). However, this region of exon 4 was not expressed in disruption mutant P2-65 and knockout mutant P2K10B (Δ*Pmcid13*), while this region of exon 4 was expressed in wild type P2 and complementation strain P265C8. The lack of expression

from this region in both P265 and P2K10B was the only loss of expression in common between these two mutant strains. The gDNA or cDNA of the 3' prime region of exon 4 did not differ between all four strains (Fig 22).

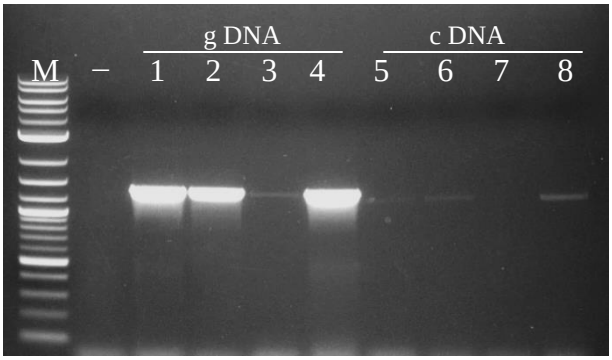

Fig 24. PCR and RT-PCR analysis of exon 1, 2 and 3 of Pm*cid13* gene. Lanes 1, 5: P2, lanes 2, 6: P265, lanes 3, 7: P2K10B, lanes 4, 8: P265C8, M: 1 kb DNA ladder (Apex) and −: negative control (water)

To determine if expression of all three 5' exons, 1, 2, and 3, of Pm*CID13* are jointly expression, PCR and RT-PCR were conducted (Fig 23). The exon 1-to-3 region was present in the gDNA of P2, P2-65 and P265C8 strongly, but only weakly amplifying from P2K10B. The expression of exons 1-to-3 was observed in the cDNA of P2, P265 and P265C8, but not observed in P2K10B.

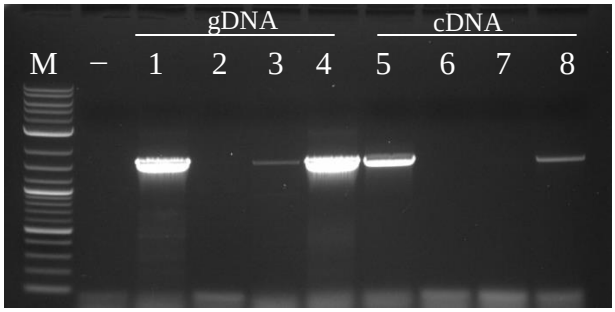

Fig 25. PCR and RT-PCR analysis of exon 3 and 5' exon4 of the Pm*cid13* gene. Lanes 1, 5: P2, lanes 2, 6: P265, lanes 3, 7: P2K10B, lanes 4, 8: P265C8, M: 1 kb DNA ladder (Apex) and −: negative control (water)

The exon 3 to 5' exon 4 region of Pmcid13 was amplified strongly from the gDNA of P2 and P265C8, very weakly from P2K10B, and not at all from P265 (Fig 24). This region was expressed in P2 and P265C8, but not in P2-65 and P2K10B, based on RT-PCR.

To investigate the expression of Pm*CID13* in pycnidia, genomic DNA and RNA were isolated from alfalfa stems colonized pycnidia. It was expected that Pm*CID13* expression from these isolated pycnidia would differ from that observed in YPS cultures. However, the expression of all exons of Pm*CID13* individually appeared to be identical in alfafa stem pycinidia and YPS cultures using all strains (data not show).

Identification of ribonulceotide reductase (RNR) in *Phoma medicaginis*

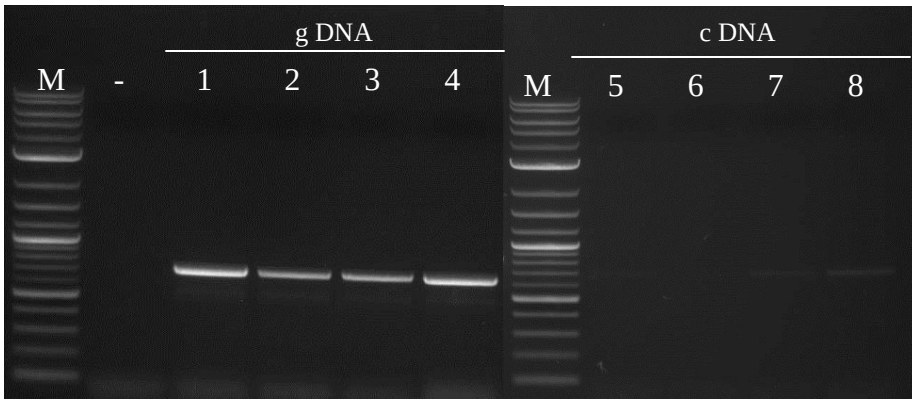

Fig 26. PCR and RT-PCR analysis of small subunit of RNR. A: lane 1: genomic DNA of P2, lane 2: genomic DNA of P265, lane 3: genomic DNA of P2K10B, lane 4: genomic DNA of P265C8, B: lane 1: complementary DNA of P2, lane 2: complementary DNA of P265, lane 3: complementary DNA of P2K10B, lane 4: complementary DNA of P265C8 M: 1 kb DNA ladder (Apex) and -: negative control (water)

In *S. pombe*, the *cid 13* gene regulates the transcription of the *suc22* gene, which encodes the small subunit (SSU) of RNR. To determine this regulation of RNR expression occurs in *P. medicaginis*, PCR and RT-PCR was conducted using primers of amplifying the orthologous SSU of RNR. Primers were designed to amplify the RNR SSU and large subunit (LSU), based on an alignment of the dothideomycete genes annotated as the RNR subunits. The *P. medicaginis* RNR SSU was amplified from the genomic DNA of the four strains, P2, P2-65, P2K10B and P265C8 (Fig 25). However, RT-PCR showed RNR SSU was expressed in P2, P2K10B and P265C8, but not P265. Additionally, the RNR LSU was found to be expressed in all strains (data not shown).

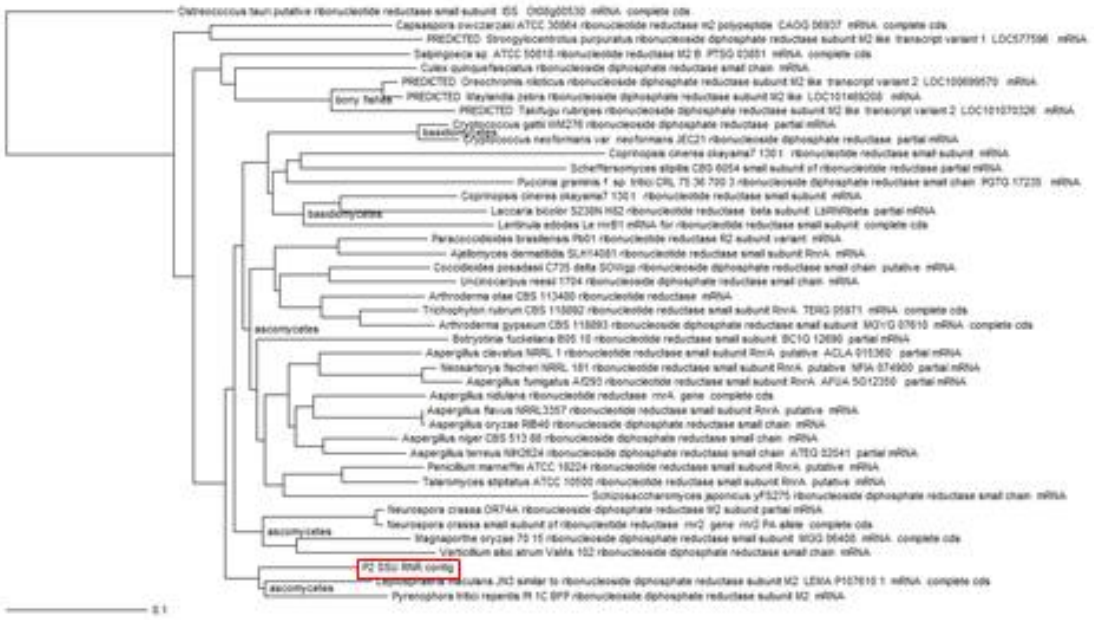

Fig 27. Blast phylogenetic analysis of RNR SSU from *Phoma medicaginis*.

In order to confirm the amplified RNR SSU was the *P. medicaginis* ortholog of the RNR subunit, PCR products were sequenced and found to be most similar to the RNR SSU of the closely related fungi, *Leptosphaeria maculans* and *Pyrenophora tritici-repentis*. (Fig 23).

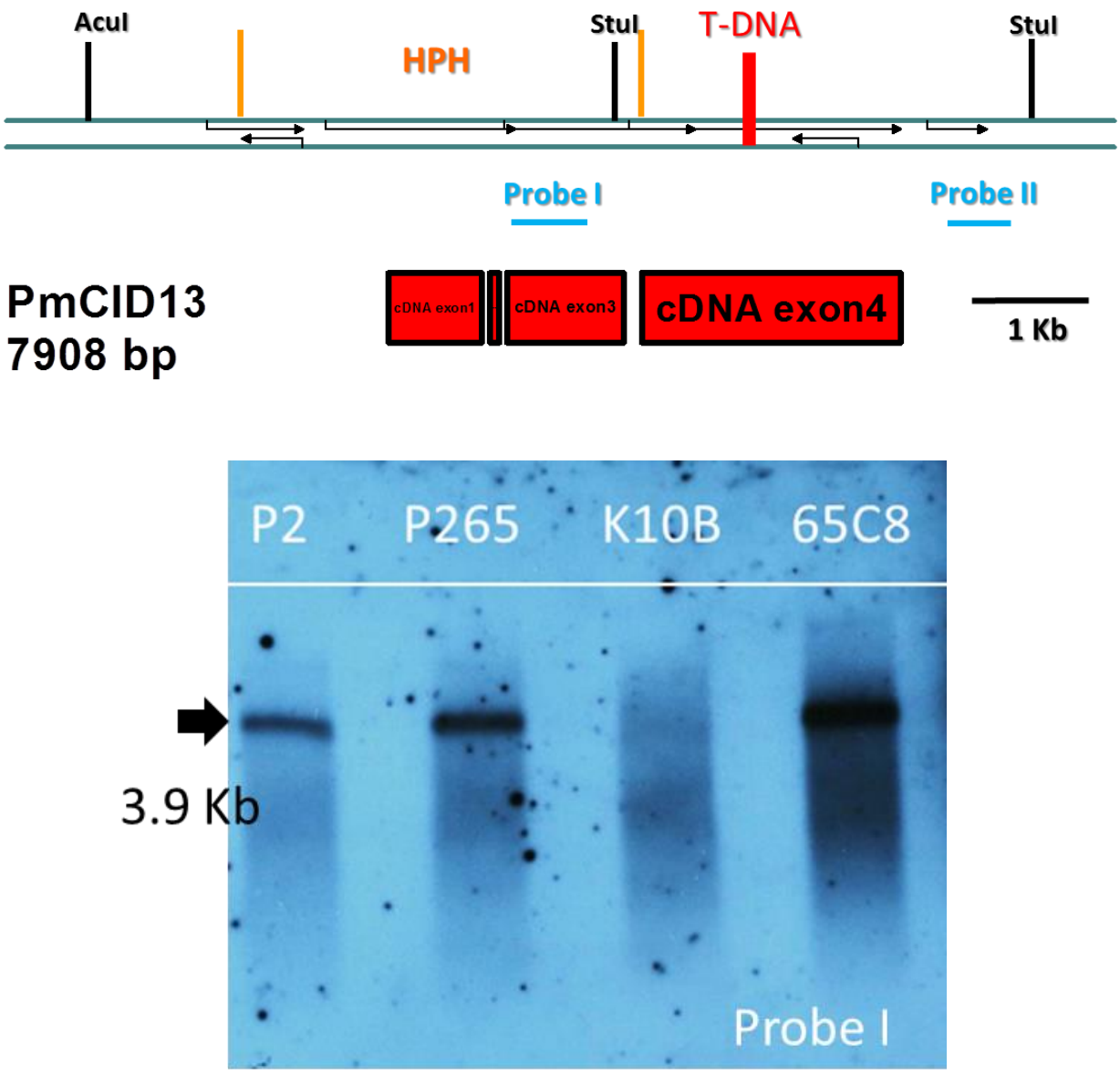

Fig 28. Verification of knockout and complement of *Pmcid13* by Southern blotting A: Schematic representation of *Acu*I and *Stu*I restriction enzyme sties and disruption by T-DNA in P265 and deletion by pPmcid13-hph in P2K10B sites. B: Southern blot analysis of the wild type and transformants using deleted region of Pm*cid13* probe. Genomic DNA was digested with AcuI and StuI prior to blotting.

The knockout and complementation mutants were analyzed by Southern hybridization (Fig 24). The deleted exon 3 of PmCID13 in knockout mutant (P2K10B) was used as

probe I. The targeted deletion of this region was confirmed by the absence of a band, while single copies of exon 3 were observed in P2, P2-65, and P265C8, using probe I. The size of the detected *Acu*I and *Stu*I fragment was estimated at 3.7 Kb.

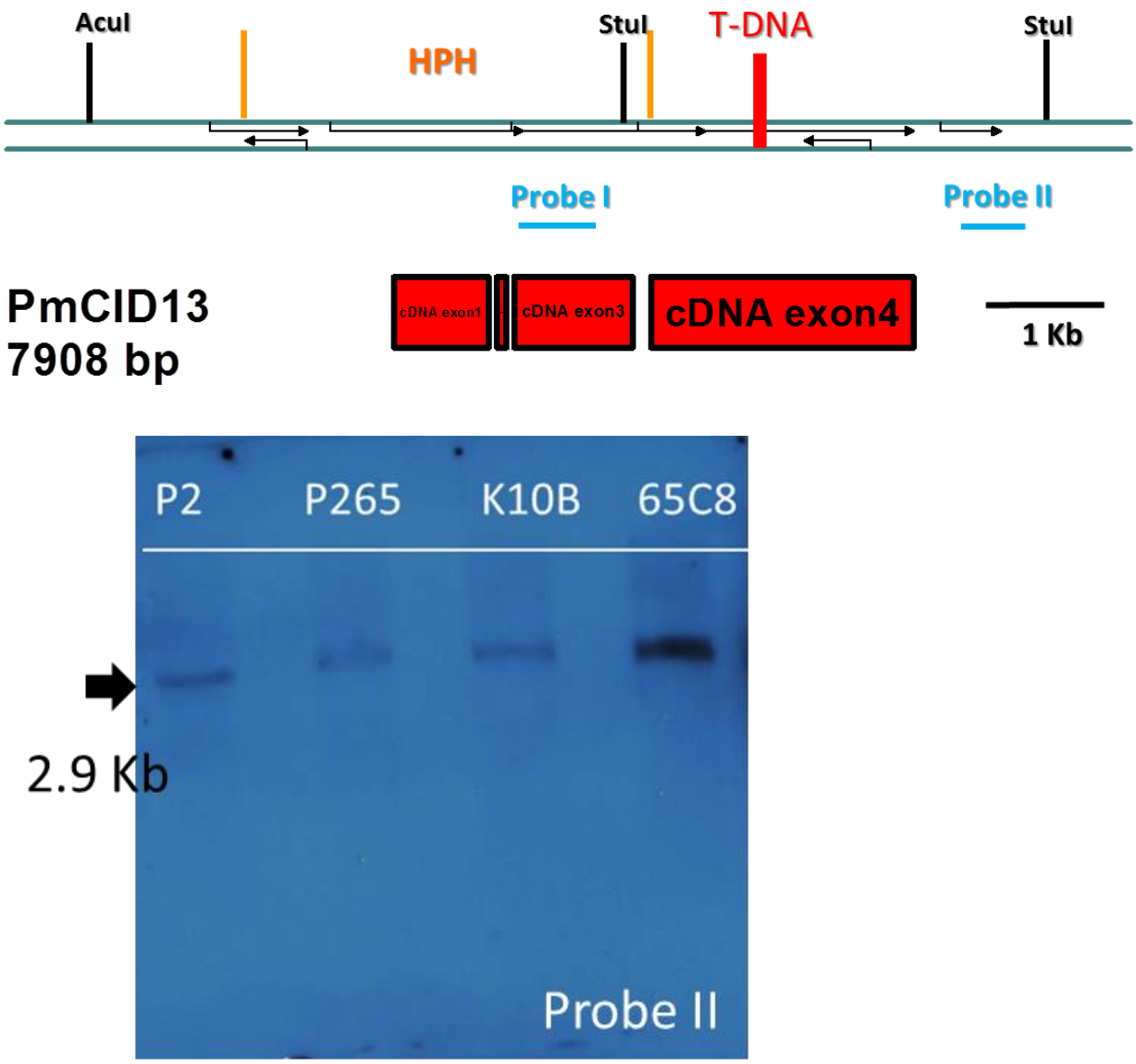

Fig 29. Verification of knockout and complement of *Pmcid13* by Southern blotting A: Schematic representation of *Acu*I and *Stu*I restriction enzyme sties and disruption by T-DNA in P265 and deletion by pPmcid13-hph in P2K10B sites. B: Southern blot analysis of the wild type and transformants using 3' flanking DNA of Pm*cid13* probe

The 3' flanking region of Pm*CID13* was used for second hybridization. In all strains, the fragment between 6649 and 7293 bp of Pm*CID13* with *Acu* I and *Stu* I was observed by

probe II. The expected sizes of P2, P2-54, P2K10B and P265C8 were 3.9, 4.1, 4.0 and 3.9 Kb, respectively.

Virulence Assays of wild-type and transformants

The virulence of wild type P2 and the Pm*cid13* mutant and complementation transformants was assessed. Disease symptoms on leaves, petioles and stems of *P. medicaginis* were evaluated at 20, 27 and 34 days post inoculation. Disease severity increased over the 3 week evaluation, but no differences in virulence among strains could be discerned.

Table 8. Mean disease severity scores of *Medicago sativa* L. 'Vernal' inoculated with conidia of strains P2, P265, P2K10B and P265C8 after 20, 27, and 34 days post inoculation (dpi).

Strains	**Disease Severity Scores**		
	20 dpi	**27 dpi**	**Day 34 dpi**
Control	0	0	0
P2	1.45 ± 0.83	1.66 ± 1.01	2.03 ± 1.64
P265	1.38 ± 0.71	1.62 ± 1.16	2.09 ± 1.76
P2K10B	1.32 ± 0.67	1.43 ± 0.90	1.71 ± 1.46
P265C8	1.14 ± 0.43	1.40 ± 0.82	1.83 ± 1.53

Discussion

Selection of transformants using antibiotic marker gene: The pycnidial mutant, P2-65, originally was transformed by a single T-DNA from binary vector pPTGFPH (Dhulipala, 2007). This T-DNA in P265 contains three genetic markers, hygromycin phosphotransferase (*hph*), neomycin phosphotransferase (*nptII*) and green florescent protein (*gfp*), and disrupts the gene, Pm*cid13*, altering pycnidial and cultural morphology. To test whether Pm*CID13* is involved in the formation of pycnidia, the Pm*cid13::TDNA* in P2-65 was complemented using the complementation vector, pCAM-Nat1-cid13,

which confers nouseothricin (Ntc) resistance by the Ntc acetyltransferase (*nat1*) gene. This complementation strategy has bene used previously. To replace the A*cveA* gene in *Acremonium chrysogenum* using *hph* gene, knockout vector pVEAKO was used for transformation. For complementation of the deleted A*cveA* gene, pG-Nat1 carrying A*cVEA* and the *nat1* gene was used for selection of complementation mutants (Dreyer et al., 2007).

Altered phenotypes of T-DNA tagged, knockout and complementation mutant: For molecular genetics for fungi, *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been demonstrated to be an effective method in terms of stable DNA integration, simple transformation, generation of large number of transformants and single T-DNA insertion(Michielse et al., 2005). The T-DNA tagged mutant, P2-65 was generated by ATMT and selected due to its formation of smaller and fewer pycnidia and more aerial hyphae compared to wild type, P2 (Fig. 1). T-DNA tagged insertional mutants have been employed to study genetic mechanisms in other plant pathogenic fungi. To identify genes important to pathogenicity in *Verticillium dahlia*, *V. dahliae* mutants were generated by ATMT and screened based on cultural phenotype, especially the formation of microsclerotia (Maruthachalam et al., 2011). In *Leptosphaeria maculans*, T-DNA insertional mutants showed altered conidial germination, morphology of sporulation, cell wall integrity, and numbers of pycnidia (Van de Wouw et al., 2009). To confirmed the involvement of Pm*Cid13* in the mutant phenotype of P265, gene deletion (ΔPm*cid13*) strain P2K10B and complementation (Pm*cid13*/Pm*CID13*) strain P265C8 were produced by homologous recombination using ATMT. However, the

cultural phenotypes (on YPS, SMM and PMM) of P2K10B and P265C8 are not indentical to those of P2-65 and wild type P2, respectively. Such partial complementation of random insertional mutant phenotypes has been documented, previously. For example, the complemented *Aspergillus fumigatus* mutants in calcineurin A (*cnaA*), which is involved in filamentous growth, did not recovered filamentous growth identical to the wild type (Steinbach et al., 2006). In *L. maculans*, the cultural phenotype of Δ*LmIFRD::TDNA* strain of was totally dissimilar to *LmIFRD* RNAi-silenced mutant in terms of pycnidial production. The T-DNA tagged mutant produced fewer pycnidia than RNAi-silenced mutant (Van de Wouw et al., 2009).

Sensitivity of mutants to HU: In this study, the Pm*cid13* disruption mutant, P265, was more sensitive to 1 mM HU than wild type. In the fission yeast, *Schizosaccharomyces pombe*, the growth of a Δ*cid13* mutant was inhibited on agar media with 10 mM HU (Saitoh et al., 2002). With 10 mM in liquid media, cell division of Δ*cid13* cells was retarded within 4 hr and survived at least 8 hr. Also, overexpression of *CID13* in *Sc. pombe* resulted in increasing resistance to HU. As with P265, the ΔPm*cid13* strain P2K10B was more sensitive to 1 mM HU, than wild type P2, which was tolerant. Against the expectation, complementation strain P265C8 was observed identical sensitivity of *Pmcid13* mutated strains (data not shown). This phenotype with HU indicated that partially complemented of *Pmcid13* in complementation strain show inconsistent with wild type, P2. In *Sc. pombe*, a multicopy plasmid carrying *CID13* partially recovered HU resistance in Δ*cid13* mutant of (Saitoh et al., 2002) .

Sensitivity of RNR to HU and DNA synthesis inhibitors: HU has long been studied as antineoplastic drug and is prescribed as a cancer chemotherapeutic (Saitoh et al., 2002). HU inhibits the production of deoxynucleotides by inhibiting the activation of ribonucelotide reductase (RNR) through scavenging of tyrosyl free radicals (Platt, 2008). The expression of RNR regulatory subunit Sml1 decreased with HU treatment. Also, RNR genes are up-regulated during S phase and after HU treatment in yeast (Elledge et al., 1992). The expression of both *cdc*22 and *suc*22, which encode subunits of RNR, are elevated in cells of *S. pombe* by treatment with HU (Sarabia et al., 1993).

Both Pm*cid13* mutants P265 and P2K10B showed sensitivity to 5 mM caffeine on YPS (Fig.). Likewise, the non-canonical PAP gene, *cid1* mutants were more sensitive to HU when combined with caffeine as an inhibitor of the fission yeast kinase Rad3 (Saitoh et al., 2002). In spite of only caffeine treatment, the Pm*cid13* mutated strains were sensitive to caffeine treatment without HU (Fig. 5).

Gallium (III) nitrate has been used for treatment of the hypercalcemia of malignancy (Apseloff, 1999). Gallium inhibits replicative DNA synthesis by activating ribonucleotide reductase (RNR) (Manfait and Collery, 1984). Moreover, the gallium-transferrin complex inhibits DNA synthesis by interacting with small subunit of the RNR (Hedley et al., 1988). The higher sensitivity of Pm*cid13* knockout strain (P2K10B) to gallium III nitrate suggests P2K10B strain is also affected in the function of RNR.

The compound 5-fluorouracil (5-fu) has been used to treat various kinds of cancers through inhibition of thymidylate synthase or incorporation into RNA and DNA, both of which inhibit RNR function (Fukushima et al., 2001; Wyatt and Wilson III, 2009). In this

study, the sensitivity of P2K10B to 5-FU was identical, suggesting similar mechanisms of action may occur in *P. medicaginis.*

Further sensitivity tests with the DNA synthesis inhibitors, cytarabine and gallium III nitrate, showed no significant differences between wild type and Pm*cid13* mutated strains. Also, sensitivities of P265 (Pm*cid13::TDNA*) and P2K10B (Pm*cid13::hph*) to DNA sysnthesis inhibitors were not identical. Only P2K10B was more sensitive to chloroquine and 5-fluoreuracil. Thus, it is tempting to speculate that the longer deleted region (exons 1-4Δ) in P2K10B than the disrupted 5' exon 4 of Pm*cid13* in P265, resulted in the more severe mutant phenotypes.

Ortholog of PAP in filamentous fungi: Canonical poly(A) RNA polymerase (PAP) performs the important role of polyadenylation of nonspecific mRNA, in the nucleus; whereas non-canonical (nc)PAPs polyadenylate specific mRNAs in both the nucleus and cytoplasm (Stevenson and Norbury, 2006). There are 24 known orthologs of the *cid13* gene from *S. pombe*. Also, the other PAP genes (Cid1, Cid12, Cid14 and Cid16) in *S. pombe* have been identified in most fungi, nematode and animal. The *cid13* gene in *S. pombe* regulates dNTPs pools by polyadenlylation of the transcript of small subunit of RNR, thereby prolonging expression (Saitoh et al., 2002; Stevenson and Norbury, 2006). The phylogenetic analysis of the cid13-like PAP gene of *P. medicaginis* indicates this gene is highly conserved across the Ascomycota (data not published). In wild type P2 and complementation strain P265C8, all four exons were transcribed. The knockout strain, P2K10B (ΔPm*cid13*) did not express exons 1, 2, and 3 of the Pm*cid13* gene, while in P265 (Pm*cid13::TDNA*) all three of these exons were transcribed (Fig 19 and 20). The

5’ region of exon 4 was not transcribed in both Pm*cid13* mutated strains, P265 and P2K10B, while the 3’ region of exon 4 was expressed in both. Therefore, the dissimilar Pm*cid13* expression profiles between P265 and P2K10B likely resulted in the nonidentical phenotypes on the media.

*Exon 1, 2 and 3 of Pm*cid13 *were involved in expression of SSU of RNR*: When cells of fission and budding yeasts incur DNA damage, dNTPs pools are induced 7-fold in *S. cerevisiae* and 2-fold in *S. pombe* (Chabes et al., 2003; Håkansson et al., 2006). In fission yeast, under DNA replication stress, Caf1 causes the nuclear Suc22 and Spd1 complex to disassemble, resulting in the degradation of Spd1 and causing Suc22 to translocate into the cytoplasm and combine with Cdc22, forming active RNR (Fig 26; (Takahashi et al., 2007). Budding yeast’s activation of RNR in response to DNA damage differs slightly from that in fission yeast. In budding yeast, Mec1 and Rad53 are activated by DNA damage or DNA replication block, inducing the transcription of *RNR* genes through up-regulation of transcriptional activator Dun1 (Fig 27). Elevated expression of RNR results in increasing dNTP pools (Zhao et al., 1998).

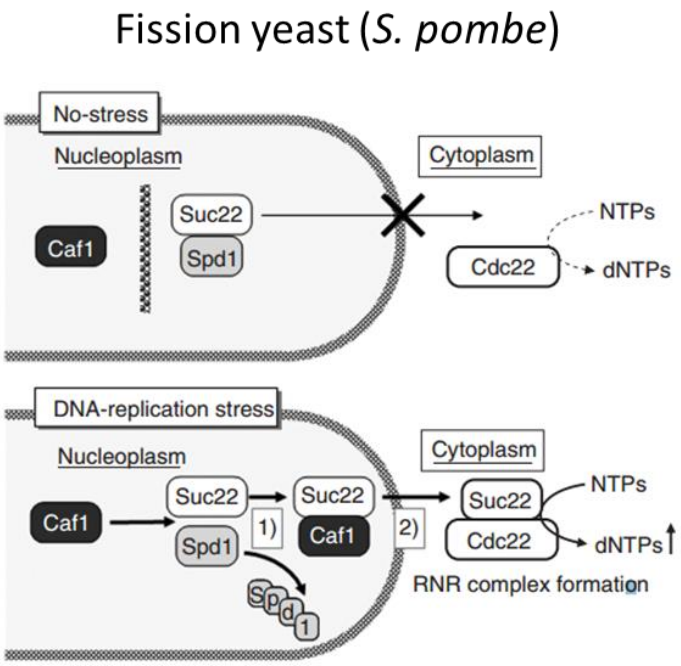

Fig 30. Possible model of translocation of Suc22 under DNA-replication stress

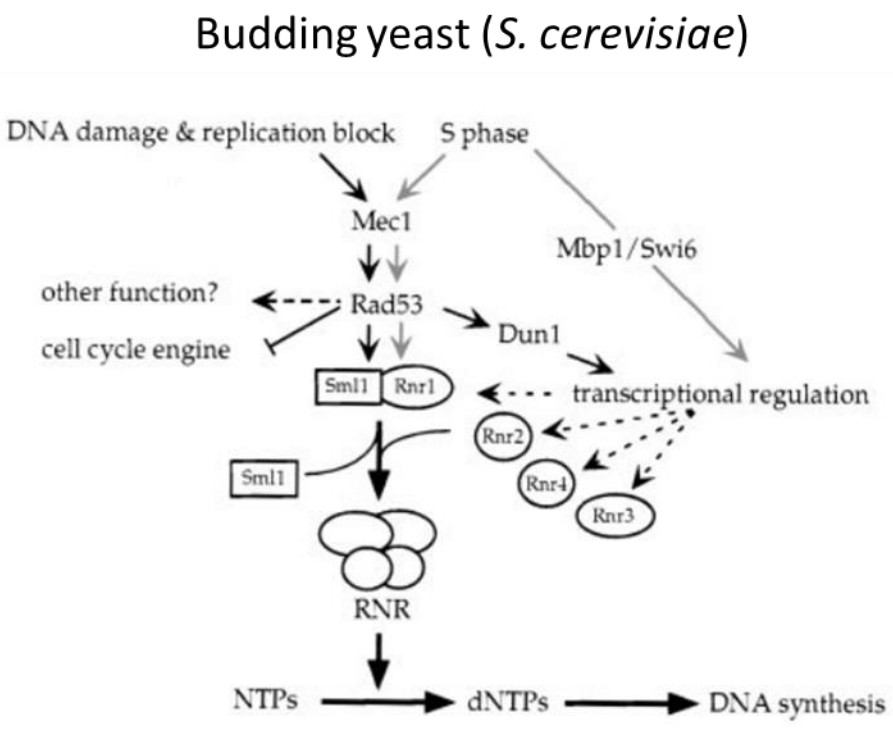

Fig 31. A model for Mec1 and Rad53 function for RNR regulation

Based on the RT-PCR gene expression results in this study, exons 1, 2 and 3 of Pm*cid13* are involved in the transcription of putative *suc22*-like small subunit of RNR in *P. medicaginis*. Double deletions of *suc22* and *cdc22* genes are lethal in *S. pombe* (Sarabia et al., 1993). However, deletion of the ncPAP *cid13*, which potentiates *suc22* expression, is not directly lethal. Similarly, disruption or deletion of Pm*cid13*, in P265 and P2K10B is not lethal. Both are able to grow on culture media and alfalfa stems. The putative (*suc22*-like) small subunit of RNR of *P. medicaginis* apparently was not expressed in P265, but expressed in all other strains. On the other hand, the putative large subunit of RNR appeared to be constitutively expressed in all strains. Differences in the expression of these possible RNR subunits may be more pronounced under replication stress, such as that induced by HU treatment.

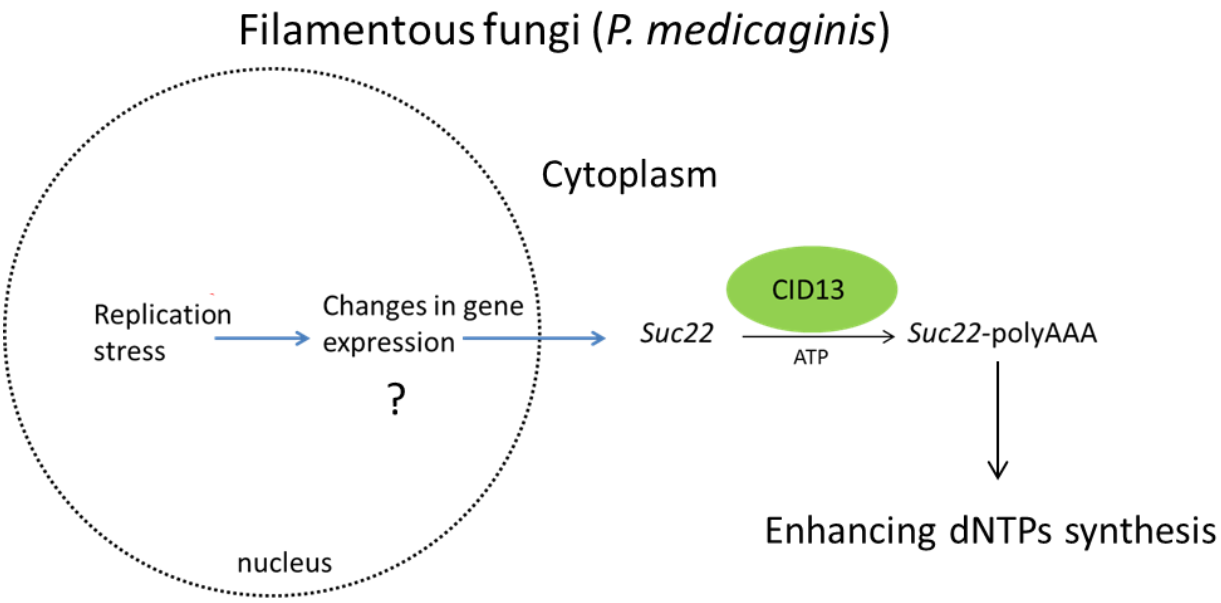

Fig 32. Possible model of regulation of dNTPs synthesis by CID13

The loss of expression of the 5' region of exon 4 of Pm*cid13* in disruption mutant P265, while maintained expression of exons 1-3, may cause the observed loss of expression of the putative RNR small subunit.

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CHAPTER V

CHARACTERIZATION OF T-DNA TAGGED MUTANT OF *PHOMA MEDICAGINIS* DEVELOPING NON-MELANIZED PYCNIDIA

Abstract

Phoma medicaginis causes spring black stem and leaf spot of alfalfa and the model legume *Medicago truncatula*. *Phoma medicaginis* produces uninucleate conidia in melanized pycnidia and is genetically tractable through *Agrobacterium tumefaciens*-mediated transformation (ATMT), which can result in insertional mutants. One such T-DNA-tagged mutant, P1A17, produced conidia in non-melanized (hyaline) pycnidia. Pycnidial melanization recovered if the mutant was supplemented with phenoloxidase substrates or allowed to age. DNA sequences flanking the insertion did not predict any disrupted open reading frames (ORF) unless a FGENESH *Coccidioides*-trained prediction algorithm was used. This *Coccidioides*-predicted ORF (*CPO*) was expressed in the wild type P1 strain, but not in the T-DNA disrupted mutant P1A17. *CPO* has not been annotated in any genomes, to date. A Δ*cpo* knockout mutant was generated by ATMT with the pCBCPO_hph vector and the knockout strain P1K29 was selected for further research. Expression of two conserved genes flanking the T-DNA-disrupted *cpo* was unchanged from the wild type. Complementation of *cpo* mutants with wild type *CPO*

partially recovered pycnidial melanization and *CPO* expression. CPO appears to be a novel regulator of pycnidium-specific melanization.

Introduction

In a previous study, the *P. medicaginis* transformant, P1A17, was observed producing non-melanized pycnidia, compared to the typical melanized pycnidia formed by the wild type P1 on the media and alfalfa stems (Dhulipala, 2007). P1A17 contains a single copy of the T-DNA from pBHt2 (Mullins et al. 2001) inserted in the genomic DNA between genes for a conserved serine-threonine protein kinase RAN1 and a conserved hypothetical protein (CHP). Pycnidial melanization of this hyaline pycnidial mutant was restored by chemical supplementation of YPS medium with the melanin precursor analogs 1,8 diaminonaphthalene (DAN) or L-DOPA (Fig 2). This indicated that the penultimate step (phenol oxidase) in the melanin synthesis pathway had not been disrupted by the T-DNA.

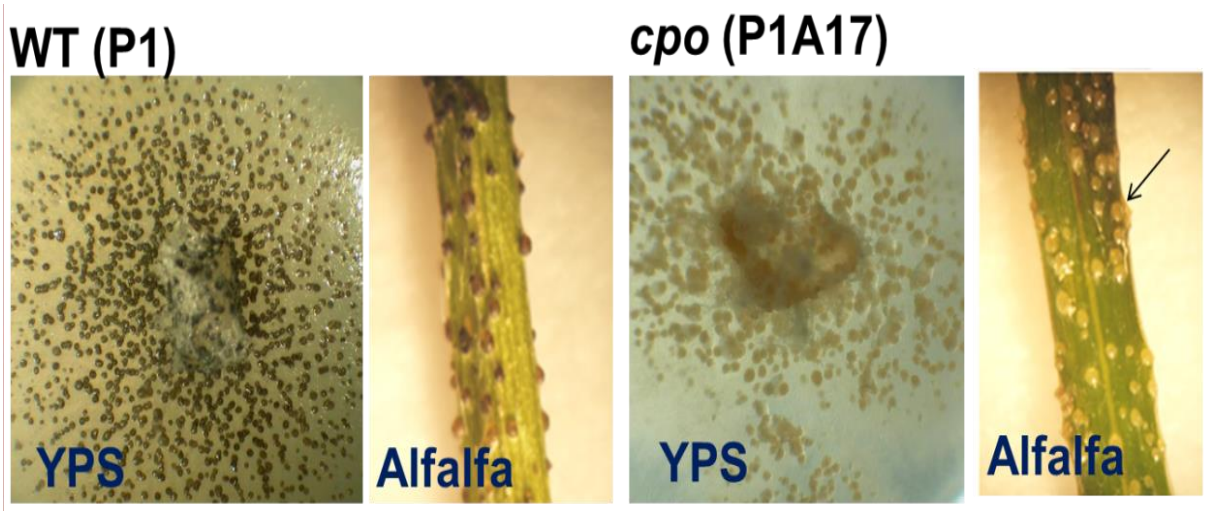


Fig 1. Phenotype of wild type (WT) strain P1 and T-DNA tagged mutant P1A17on YPS media, 14 days post inoculation (dpi) and on alfalfa stems, 7 dpi. Non-melanized pycnidia of P1A17 were observed on both YPS and alfalfa (arrow).

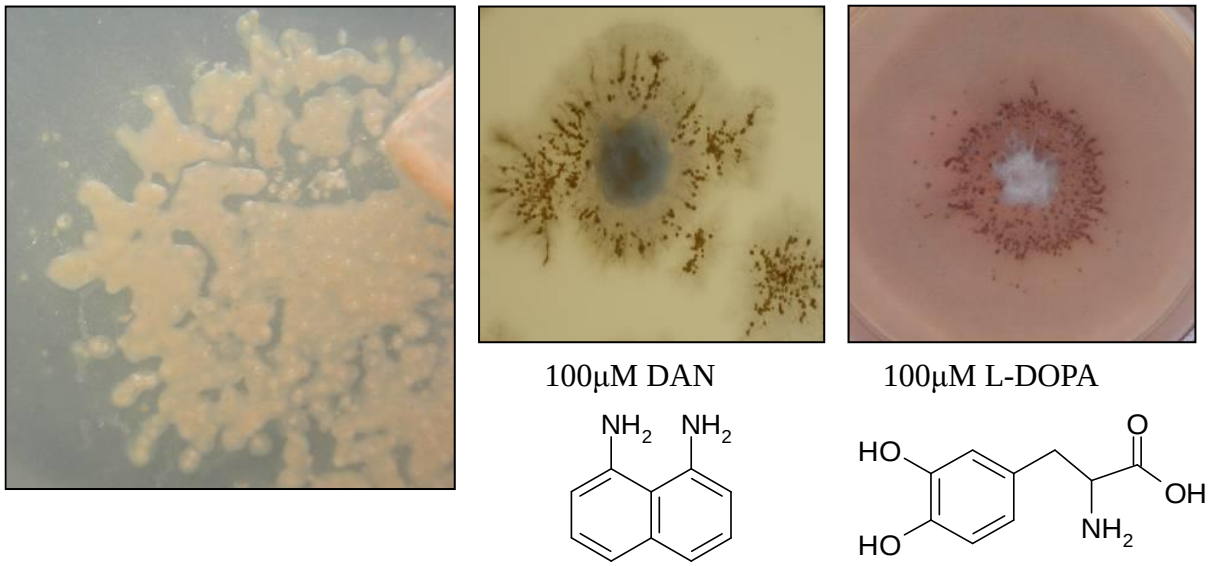


Fig 2. Chemical complementation of P1A17 through supplementation with melanin precursor analogs, 1,8 diaminonaphthalene (DAN) or L-3,4-dihydroxyphenylalanine (L-DOPA). Hyaline pycnidia producing conidia in peach-colored matrix (left) and melanized pycnidia formed when supplemented melanin precursor analogs (center and right).

Melanins are composed of polymerized phenolic or indolic monomers, and also usually include protein or carbohydrates. Most of melanin appears as black or near black pigments. Development of melanin is not essential part in terms of fungal growth and reproduction. However, these biological macromolecules permit fungi to survive various kinds of environmental stresses (Butler and Day, 1998).

Fungi synthesize three different melanins from three different precursors, L-dihydroxyphenylalanine (L-DOPA), glutaminyl-3,4-dihydroxybenzene (GDHB) and 1,8 dihydroxynaphthalene (DHN). While L-DOPA and GDHB melanins occur in other

organisms, DHN has been reported only in filamentous fungi (Hegnauer and Rast, 1985; Bell and Wheeler, 1986; Miranda et al., 1992).

The biosynthesis of L-DOPA melanin is catalyzed by tyrosinase or laccase via hydroxylation of L-tyrosine to dopaquinone or oxidation of L-DOPA to dopaquinone. L-DOPA is an intermediate as essential factor in the either reaction (Langfelder et al., 2003). The well characterized fungal DHN melanin is synthesized via a polyketide pathway (Fig 3). In the final step of DHN melanin synthesis, 1,8-DHN is polymerized via an oxidative reaction, likely catalyzed by a laccase or polyphenoloxidase (Butler and Day, 1998).

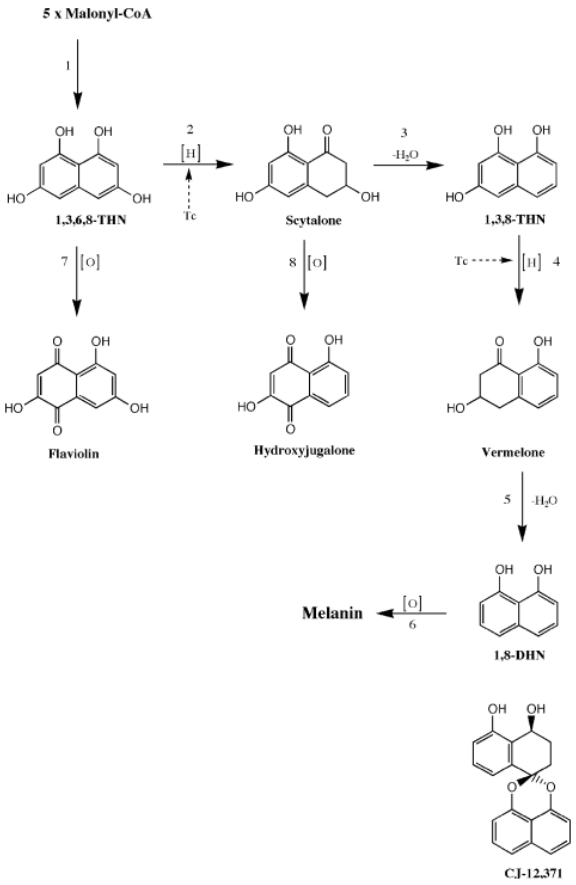


Fig 3. The polyketide pathway of fungal DHN melanin biosynthesis (adapted from Lanfelder et al., 2003). Intermediate products and inhibition of certain pathway were isolated by thin layer chromatography (TLC) or high pressure liquid chromatography (HPLC). Precursor of 1.8-DAN and L-DOPA were used for chemical complementation of P1A17.

Experimental Approach

Strains, media and culture condition: Wild-type, P1 and mutants, P1A17, P1K29 and P1A17C23 (Table 1) were cultured on YPS and MYPS (recipes in previous chapter). Liquid cultures were started using three 1 cm^2 agar plugs of *P. medicaginis* vegetative mycelium that were bead beat as described in the previously chapter. After beadbeating, 1 ml mycelial suspension was used to inoculate tissue culture flasks with vented caps (Corning, Randor, PA) containing 20 ml of MYPS. These cultures were incubated for 5 days at room temperature in the dark. For a higher yield of DNA, the culture were shield from exposure to light, resulting in the suppression of melanization, which is light induced. After 5 days, fungal mycelia were collected by vacuum filtration onto filter paper until slightly dry, washed with sterilized water and transferred to a 50 ml Falcon™ tubes. The liquid nitrogen was added immediately into Falcon™ tubes and frozen samples were stored at -80℃.

Table 1. Wild type strains and mutants used in this work.

Strains	Genetic traits[a]	Relevant characteristics
Phoma medicaginis		
P1, P2	Wild-Types	Melanized pycnidia
P1-A17	Hyg^R, *cpo::TDNA*	Hyaline pycnidia
P1K29	Hyg^R, *cpo::TDNA*	Partially hyaline pycnidia
P1A17C23	Nat^R	Partially recovered phenotype of P1
Agrobacterium tumefaciens		
AGL-1	*recA*::*bla*, Amp^R	Routine ATMT

Table 2. Plasmids used in this work

Strains	Genetic traits[a]	Relevant characteristics
ATMT plasmids		
pBHt2	Kan^R, Hyg^R	T-DNA tagging
pCB403.2	Kan^R, $G418^R$	Gene deletion vector
pCBCPO	Kan^R, $G418^R$,	Cloned *CPO* gene
pCBCPO-hph	Kan^R, $G418^R$, Δ*cpo*::hph, Hyg^R	*cpo* gene deletion vector
pCAM-Nat1	Kan^R, Ntc^R	Complementation 'empty' vector
pCAM-Nat1-CPO	Kan^R, Ntc^R, *CPO*	Complementation of *cpo*

Table 3. Primers used in this work

Primer name	Sequence (5'-3')	TM	Description
CPOFP-1F-SpeI	TAGTACTAGTGCGGGTATGTACAATGG	68.6	Make KO vector
CPOFP-6R-SbfI	CCGTCCTGCAGGGTTGATTAATGATTA	63.5	Make KO vector
pCB-CPO-F	TACCGTGACACCGTGATAC	61.6	Make KO vector
pCB-CPO-R	TGCCAGTGCAGAGATACAC	62.3	Make KO

			vector
PoliC-HPH-F	TATCTCTGCACTGGCACTATTCCTTTGCCC TCGGACG	70.8	Make KO vector
PoliC-HPH-R	CACGGTGTCACGGTACTGCAGCTGTGGAG CCGC	70.8	Make KO vector
LBP1A17conf2	GTACGTTGAGAGACGGTAGG	54.3	Confirm KO vector with Hyg-R
Hyg-R	GCCGATGCAAAGTGCCGATAAACA	60.4	Confirm KO vector with LBP1A17conf2
SbfI_CPO_com _R	GGTCCCTGCAGGGCGAATTCGAGTG	69.2	Confirm complementatio n vector and strains with XbaI_CPO_com _F
XbaI_CPO_com _F	ACGGTCTAGAGAGAGTTTACTGCTCG	62.8	Confirm complementatio n vector and strains with SbfI_CPO_com

			_R
M13F	GTAAAACGACGGCCAGT	55.2	Confirm the KO strain with Hyg-F
Hyg-F	AGAGCTTGGTTGACGGCAATTTCG	60.2	Confirm the KO strain with M13F
RT_A17LB_F	TGCACATCCAGACCAACATTCCCT	65.4	Amplify RAN1 region with RT_A17LB1_R
RT_A17LB1_R	TGGCAGGGTTGAAATCAAAGACGC	63.7	Amplify RAN1 region with RT_A17LB_F
RT_A17RB1_F	ATGCAGAACTTGAGCAGCCAACAC	64.7	Amplify CHP region with RT_A17RB1_R
RT_A17RB1_R	GTTGCAGACACGAAACGATTGCGA	64.6	Amplify CHP region with RT_A17RB1_F
Cocci pred ORF-F	CCACCTACGATCCGCGGCAG	67.7	Amplify CPO region with Cocci pred

			ORF-R
Cocci pred ORF-R	TCCGTCCTTGAAGTCGGGTTCG	64.9	Amplify CPO region with Cocci pred ORF-F
CHP-muPlex-F	CCTGAGGATGAAGCTTTGAATA	58.5	Amplify CHP region with CHP-muPlex-R in multiplex PCR
CHP-muPlex-R	CAAGCTGTGTGGAGAATTTGA	58.9	Amplify CHP region with CHP-muPlex-F in multiplex PCR
CPO-muPlex-F	GGTCGAGTCCACCTACGATC	59.5	Amplify CPO region with CPO-muPlex-R in multiplex PCR
CPO-muPlex-R	GTTTCCCGCCTTCAGTTT	57.7	Amplify CPO region with CPO-muPlex-F

			in multiplex PCR
RAN1-muPlex-F	CAACCTTACTTGTGGACGCA	59.8	Amplify RAN1 region with RAN1-muPlex-R in multiplex PCR
RAN1-muPlex-R	GGTAGAGGAGCACATGGACG	60.7	Amplify RAN1 region with RAN1-muPlex-F in multiplex PCR
P2-ef1a-muPlex-F	TCCAAGGATGGCCAGACT	59.1	Amplify ef1a region with efa1-muPlex-R in multiplex PCR
P2-ef1a-muplex-R	GGAGGGCACCATCTTGAC	59.0	Amplify ef1a region with efa1-muPlex-F in multiplex PCR

Construction of complementation and knockout vector: In order to construct a Δ*cpo* knockout vector, the *CPO* gene region (~3 Kb) was amplified from *P. medicaginis* P1 with primers (CPOFP-1F-SpeI and CPOFP-6R-SbfI) containing SbfI and SpeI sites and cloned into these sites on binary vector pCB403.2, resulting in the vector pCBCPO. The outward directed primer pair pCB_CPO forward and pCB_CPO reverse was used to amplify the pCB403.2 backbone and the 1 kb upstream and downstream regions of *CPO* from P1. Simultaneously, the *PoliC::hph* cassette was amplified from pOHT using PoliC_hph forward and PoilC_hph reverse primers. These two fragments assembled into the knockout vector pCBCPO-hph using a Gibson Assembly Kit (New England BioLabs, Ipswich, MA) according to company's instruction. Briefly, the DNA fragments *PoliC::hph* and pCBCPO were amplified with primers designed to have 15 and 16 bp overlaps at the junction of each amplicon and ligated using the Gibson assembly kit. The assembled knockout vector, pCBCPO-hph , was transformed and maintained in *E.coli* DH5α. The pCAM-Nat1-CPO complementation vector was constructed using pCAM-Nat1. A ~3 Kb DNA fragment containing the *CPO* gene and ~1 kb 5'- and 3'- flanking regions from P1 genomic DNA was amplified using the cpo_compl_Xba I and cpo_compl_Sbf I primer pair containing engineered *Xba*I and *Sbf*I restriction sites. This amplified *CPO* product and vector pCAM-Nat1 were digested with *Xba* I and *Sbf* I and ligated, producing the ATMT complementation vector, pCAM-Nat1-CPO, which was maintained in *E.coli* DH5α. For ATMT, each vector was transformed into *A. tumefaciens* AGL1 and ATMT of *P. medicaginis* conidia performed as described in previous chapters.

Semi-quantitative RT-PCR (sq-RT-PCR): Total RNA was isolated from cultures of P1, P1A17, Δ*cpo* deletion strains, and *cpo* complementation strains and converted to cDNA as described in Chapter IV. A sequence of elongation factor 1 alpha of the *P. medicaginis* was used as suitable housekeeping gene to serve as a positive sq-RT-PCR (Choquer et al., 2003) control. The primers used for RAN1, CHP and CPO were designed by *muPlex* (Rachlin et al., 2005). Primer sites for each gene were selected on either side of an intron, so that PCR products amplified from cDNA produced amplicons smaller in size from those produced from genomic DNA. All multiplex RT-PCRs were conducted using ef1a-muplex, RAN1-muplex, CHP-muplex and CPO-muplex forward and reverse primers in the same reaction tubes. The PCR program used was as follows: an initial denaturation at 95℃ for 5 min; 30 cycles of 95℃ for 30 s, 57℃ for 1 min and 72℃ for 1 min; followed by an additional 10 min extension at 72℃. For relative quantitation (semiquantitative), replicated RT-PCR reactions were collected after 15, 20, 25 and 30 cycles and bands separated by electrophoresis on a 1.0% TAE agarose gel, stained with ethidium bromide.

Hydroxyurea (HU) test: In order to test sensitivity to HU with wild type and *cpo* mutated strains and complementation strains, 50 ml falcon tubes containing 25 ml of 1, 2 and 5 mM of HU were used for inoculation test. The agar plugs from cultures of each strain were immersed for 30 min at various concentration of HU (1, 2 and 5 mM). The immersed plugs inoculated into YPS agar media and then incubate the inoculated plated at 20℃.

Results

Morphological characteristics of wild-type and transformants

From previous research, nine hundred eighty two transformants were generated by ATMT (Dhulipala, 2007). Most were transformed using pBHt2, an ATMT binary vector containing the hygromycin selectable marker, *PtrpC::hph*. Among these T-DNA-tagged mutants, P1A17 produced hyaline pycnidia.

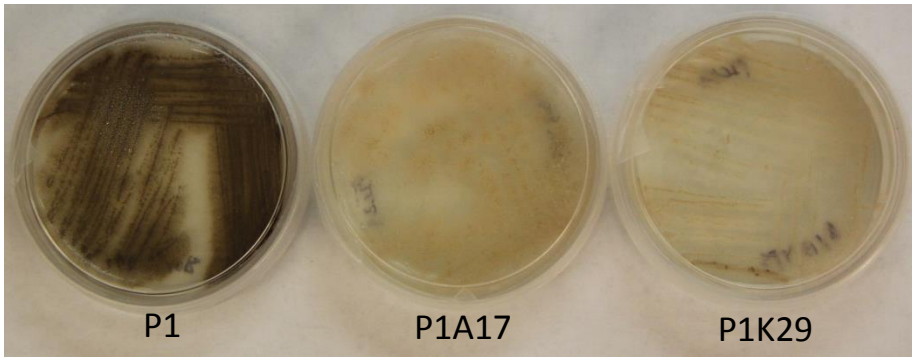


Fig 4. Phenotypes of P1 (wild type), P1A17 (*cpo::TDNA*), and P1K29 (Δ*cpo::hph*) on YPS 10 dpi. Loss of pycnidial melanization the *cpo* knockout transformant P1K29 was similar to that in in the original disruption mutant P1A17.

In order to confirm *CPO* is needed for wild type pycnidial melanization, over one hundred gene deletion (Δ*cpo*) mutant candidates were generated in *P. medicaginis* P1 using ATMT and knockout vector pCBcpo_hph. Cultural phenotypes of candidate mutants were compared to wild type P1 and T-DNA tagged mutant P1A17 on several media. Among these knockout strains, 70 candidate Δ*cpo* transformants were selected based on cultural characteristics on YPS (e.g. reduced pycnidial melanization). While

wild type strain, P1 produced melanized pycnidia on YPS, P1A17 and P1K29 displayed non-melanized pycnidia on YPS (Fig 4).

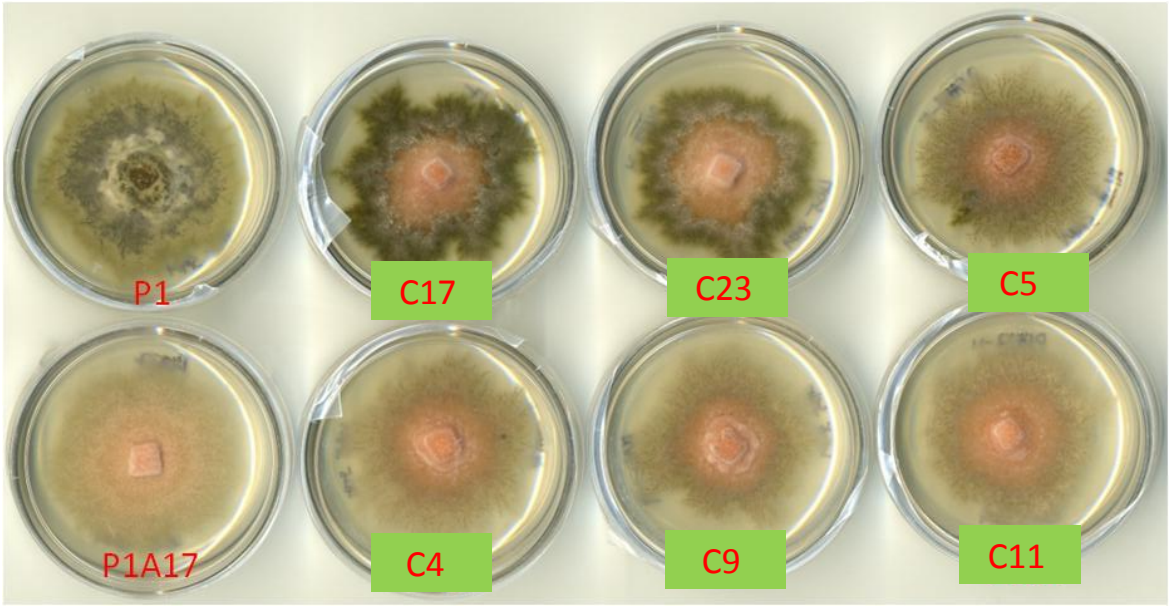


Fig 5. Phenotypes of P1 (wild type), P1A17 (*cpo::TDNA*) and complementation strains P1A17-C23 (*cpo::CPO*) on SMM 10 dpi. Partial restoration of pycnidial melanization was observed in P1A17-C17, P1C23, and C5.

In order to confirm wild type *CPO* can restore pycnidial melanization, eighty-eight candidate complementation transformants were generated in P1A17 using ATMT and complementation vector pCAM_ Nat1_CPO. Transformants were selected on YPS containing Ntc (50 ug/ml) and examined for recovery of pycnidial melanization. However, only partially restored melanization was observed in complementation transformants was observed on SMM (Fig 5). P1C23 was used for further research.

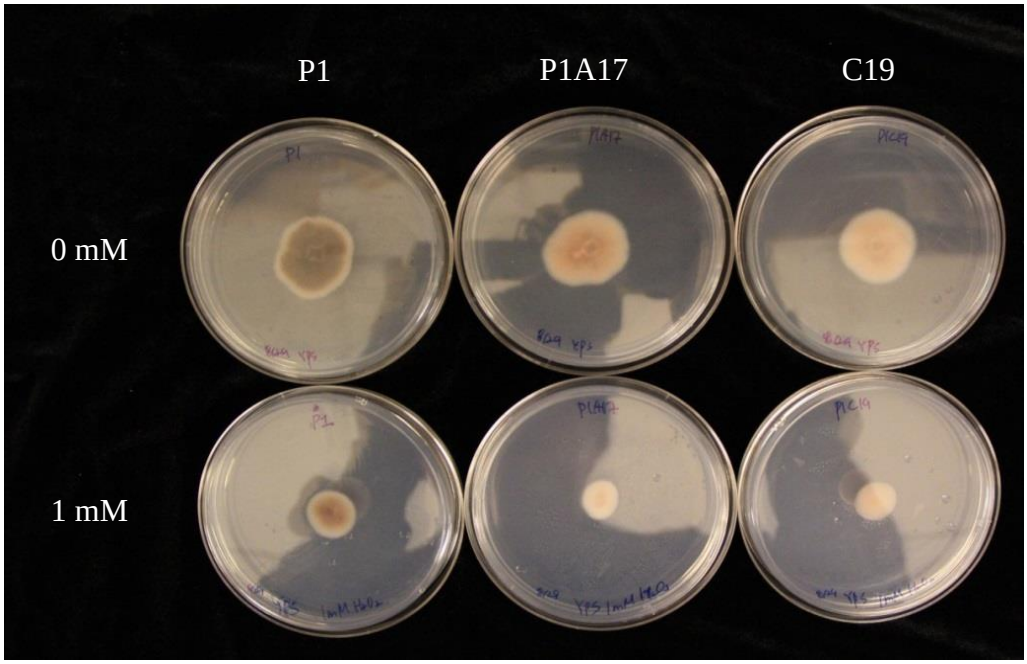


Fig 6. Wild type (P1), melanin deficient *cpo* mutant (P1A17) and complementation strain (P1A17-C19) appear to be equally sensitive to 1 mM hydrogen peroxide (H_2O_2).

Sensitivity of The melanized fungi by1,8-dihydroxynaphthalene were more resistant to H_2O_2 than alnoto stains did (Jacobson et al., 1995). Therefore, we hypothesized that non-melanized strains show more sensitive to H_2O_2. However, all strains retained to grow on YPS containing 1 mM of hydrogen peroxide.

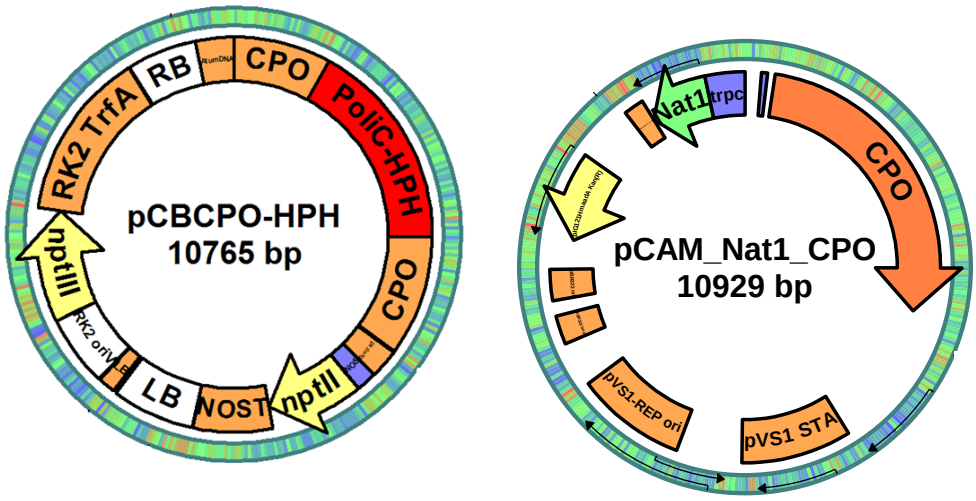


Fig 7. Construction of *cpo* gene knockout and complemention vectors, pCBCPO_hph and pCAM_Nat1_cpo used for *cpo* deletion and complementation of *P. medicaginis* P1

Construction of *cpo* knockout and complementation vectors

To identify the role of *cpo* gene in *P. medicaginis*, the pCB_cpo_hph knockout vector (Fig 4) was constructed with the backbone of pCB403.2 *Agrobacterium* binary vector. Also, the pCAM-Nat1-CPO complementation vector (Fig 7) was constructed using pCAMBIA 1300 (CAMBIA, Canberra, Australia). The knockout vector (pCB_cpo_hph) has ~7.0 Kb of T-DNA including 5' and 3' flanking of *CPO* and PoliC promoter and hygromhycin phosphotransferase fragement. The complementation vector (pCAM-Nat1-CPO) has ~4.6 Kb of T-DNA containing the *CPO* gene and ~1 kb 5'- and 3'- flanking regions from P1 genomic DNA.

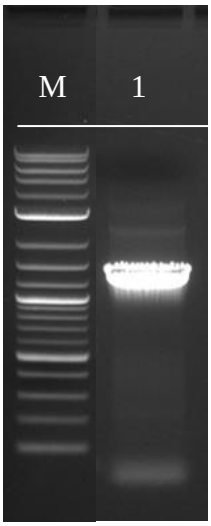


Fig 8. PCR agarose gel confirmation of knockout vector, pCBCPO_hph
Lane 1: pCBCPO-hph and M: 1 kb DNA ladder (Apex)

The knockout vector, pCB_cpo_hph was constructed using Gibson assembly, as described above in the Materials and Methods, and was verified by PCR amplification of the multiple cloning site, OHT cassette, and 3' flanking DNA of *cpo,* using primers M13 forward and reverse, LBP1A17conf2, located in 3' flanking DNA of *cpo,* and hph reverse primer in OHT (Fig 8) and by sequencing (data not shown).

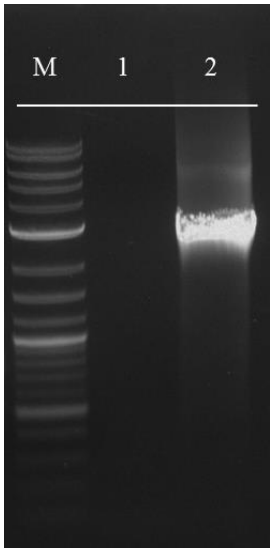


Fig 9. Agarose gel of PCR confirmation of complementation vector, pCAM_Nat1_CPO Lane 1: pCAM-Nat1, lane 2: pCAM-Nat1-cpo and M: 1 kb DNA ladder (Apex). The amplicon in the lane 2 include predicted *CPO* ORF with ~1 Kb upstream and downstream of CPO.

In order to complement the T-DNA-disrupted *cpo* ORF (*cpo::TDNA*) in P1A17, the complementation vector pCAM-Nat1-CPO, containing the predicted *CPO* open reading frame with ~1 kb upstream and downstream sequences, was constructed and verified by PCR (Fig 9) and sequencing (data not shown).

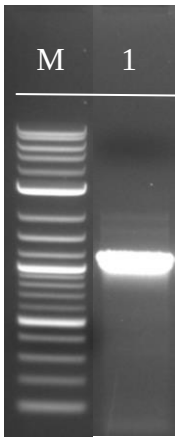


Fig 10. Agarose gel of PCR confirmation of candidate Δ*cpo* knockout transformants. Lane 1: Δ*cpo* transformants, P1K29 and M: 1 kb DNA ladder marker (Apex)

Analysis of *cpo* knockout and complementation transformants

To assess the role of the *CPO* gene, P1 was transformed using pCB_cpo_hph create a gene knockout (Δ*cpo*) and P1A17 was transformed using pCAM-Nat1-cpo to complement the disrupted *cpo* gene. After transformation, 70 candidate knockout transformants were selected on YPS containing hygromycin (50 ug/ml) and transformants with less melanized pycnidia selected (e.g. Fig 4) and T-DNA intregration confirmed by PCR (Fig 10).

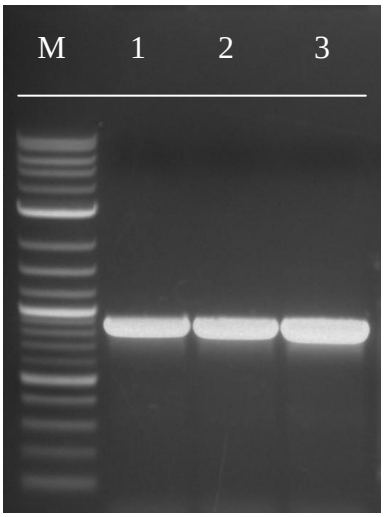


Fig 11. Agarose gel of PCR confirmation of candidate *cpo/CPO* complementation transformants by PCR. Lane 1: P1, lane 2: P1A17C7, lane 3: P1A17C23 and M: 1 kb DNA ladder marker (Apex)

To determine whether the predicted wild type *CPO* would restore pycnidial melanization in P1A17, the wild type *CPO* locus from P1 was introduced by ATMT into the P1A17 using a nouseothricin resistance marker. And, 88 transformants were selected on YPS with nouseothricin (50 ug/ml) and six of the transformants were chosen based on increased pycnidial melanization (e.g. Fig 5). Two of these, P1A17C7 and P1A17C23, were PCR-positive for the occurrence of a complete *CPO* gene (Fig 11) and P1A17C23 was used for further research.

Analysis of expression of *RAN1, CHP* and *CPO* in wild type and transformants

To determine if expression of RAN1, CHP and CPO are altered in P1 and P1A17, genomic DNA (gDNA) and total RNA were isolated and complementary DNA (cDNA) was synthesized from total RNA to conduct PCR and RT-PCR, respectively, as described

above. Both *RAN1* and *CHP* were expressed similarly in P1 (wild type) and P1A17 (*cpo::TDNA*) (Fig 12).

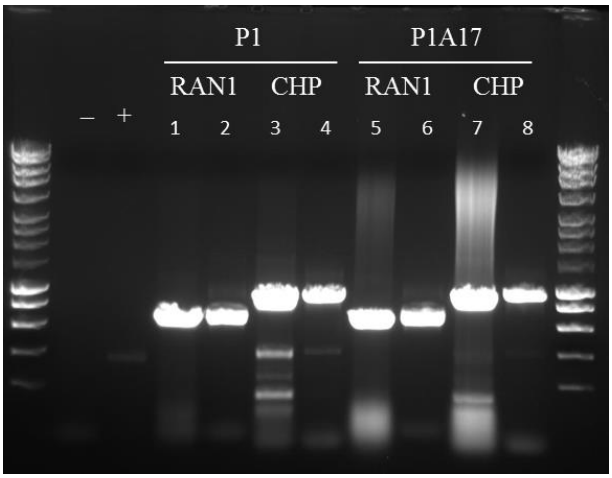


Fig 12. PCR and RT-PCR analysis of *RAN1* and *CHP* in P1 and P1A17. Lanes 1 and 3: gDNA of P1, lanes 2 and 4: cDNA of P1, lanes 5 and 7: gDNA of P1A17, and lanes 6 and 8: cDNA of P1A17.

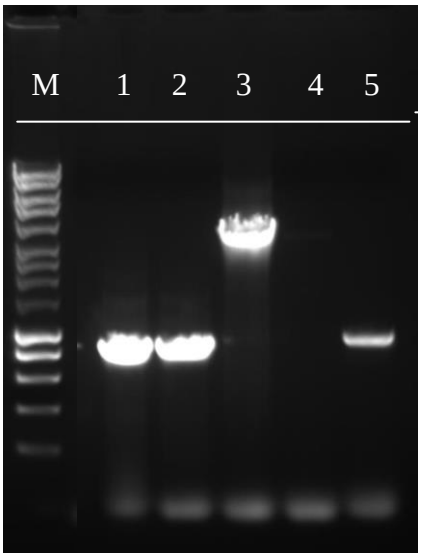


Fig 13. PCR and RT-PCR analysis of *CPO* expression in P1, P2 and P1A17. Lane 1: gDNA of P1, lane 2: gDNA of P2, lane 3: g DNA of P1A17, lane 4: cDNA of P1A17, lane 5: cDNA of P1 and M: 1 kb DNA ladder (Apex)

The *CPO* gene was amplified as a ~900 bp band from the gDNA of wild type strains P1 and P2 (Fig 13). However, the product amplified by the same primers from the gDNA of

P1A17 was ~4 Kbp, a size difference predicted due to the insertion of the T-DNA from pBHt2. Moreover, using cDNA, expression of *CPO* was observed in P1, but not in P1A17 (Fig 13), indicating the TDNA disruption of *CPO* prevented expression.

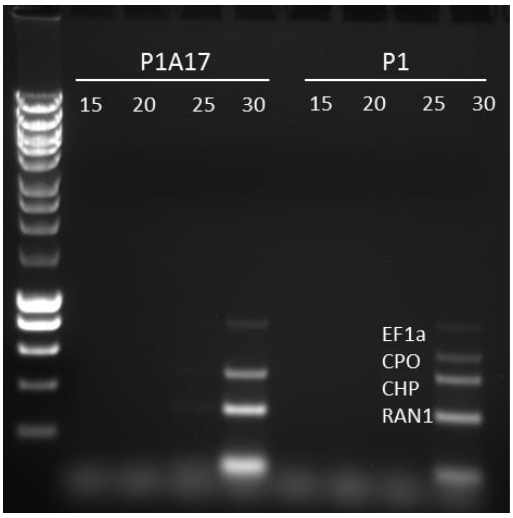


Fig 14. Multiplex semiquantitative PCR detection of *RAN1*, *CHP* and *CPO* in P1 and P1A17 using gDNA. PCRs were removed from the thermocycler at the cycle number indicated above each lane. *EF1α* was used internal PCR control.

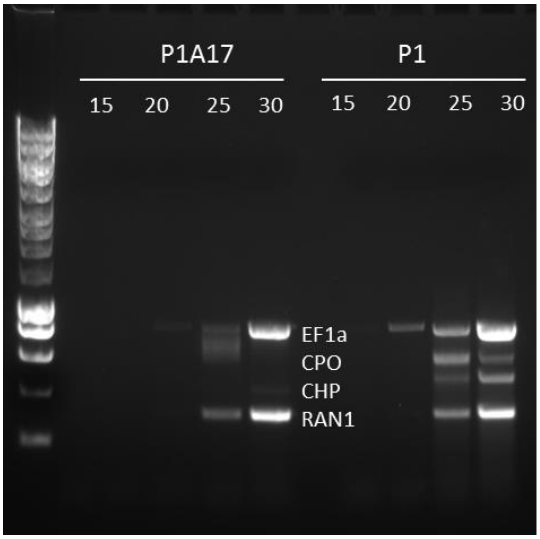
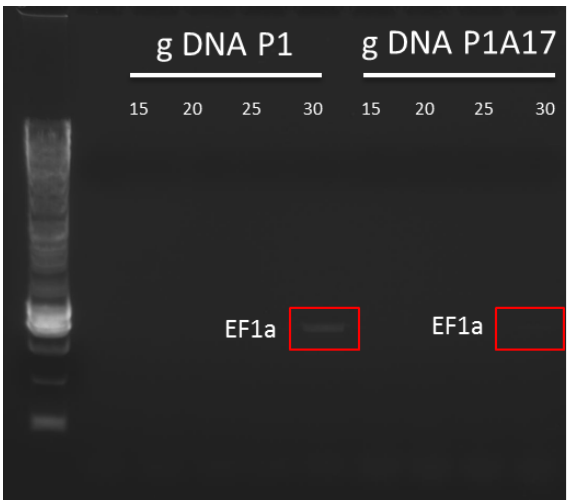


Fig 15. Multiplex semiquantitative RT-PCR detection of *RAN1*, *CHP,* and *CPO* in P1 and P1A17 using cDNA. PCRs were removed from the thermocycler at the cycle number indicated above each lane. EF1α was used internal PCR control.

To estimate the expression level of the genes surrounding the T-DNA insertion site, a 4-gene multiplex sq-RT-PCR was used (Choquer et al., 2003). To confirm multiplex PCR primers amplified the correct products, all genes were amplified by sq-PCR from the gDNA of both strains (Fig 14). Only the *cpo* gene of P1A17 was not amplified under the reaction conditions used.

For sq-RT-PCR, the internal control EF1a (constitutively expressed housekeeping gene) was observed earliest, from 20 to 30 cycles, in both strains and RAN1 was detected next in both strains, from 25 to 30 cycles (Fig 15). Differences in expression of *CPO* and *CHP* between P1 and P1A17, were observed. While both *CPO* and *CHP* were expressed in P1, *CPO* was not transcribed in P1A17 and *CHP* expression in P1A17 was much lower than in P1.

Primers and PCR condition for multiplex PCR can be challenging to optimize (Edwards and Gibbs, 1994). Therefore, uniplex sq-PCRs and sq-RT-PCRs were conducted with each primer pair (Figs 16-1) to verify the results of multiplex sq-RT-PCR. The internal control, *EF1a* was amplified from the gDNA and cDNA of both P1 and P1A17 using sqPCR and sqRT-PCR (Fig 16). Also, *RAN1* was amplified from both strains (Fig 17). These results were similar to those observed in the multiplex PCRs. Also, as observed in multiplex PCRs, *CPO* was not amplified from or transcribed in P1A17, but was present and expressed (though faint) in P1 (Fig 19). On the other hand, *CHP* was amplified from the gDNA and cDNA of both strains using uniplex PCR, indicating it is expressed (faintly) in both strains and the lack of a *CHP* band in P1A17 multiplex RT-PCRs may have been due to interference from the other primers.



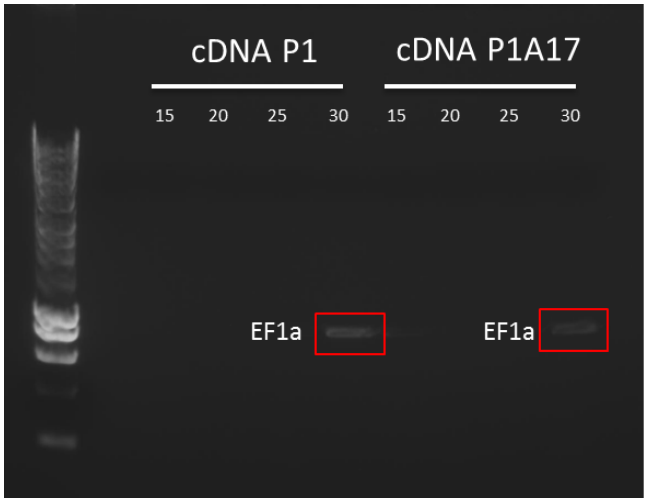
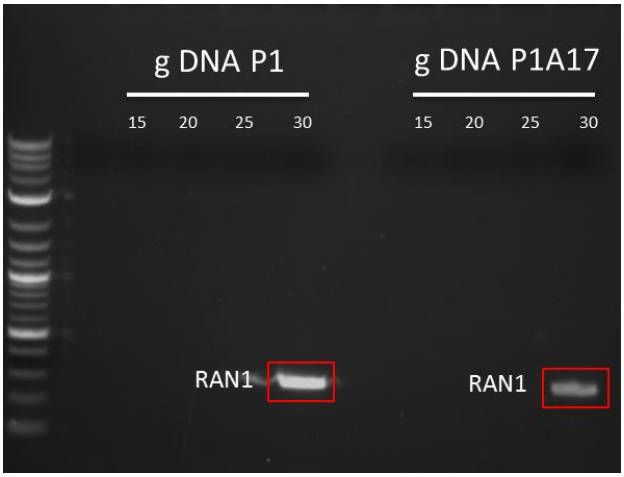


Fig 16. Uniplex sqPCR detection of *EF1a* (internal control) in P1 and P1A17 using gDNA or cDNA as templates. PCRs were removed from the thermocycler at the cycle number indicated above each lane.



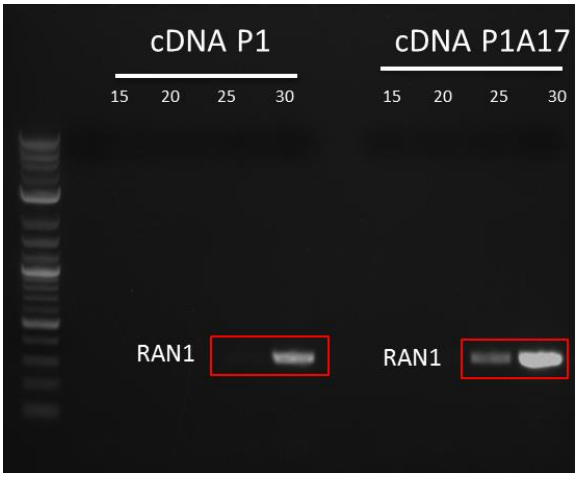


Fig 17. Uniplex sqPCR detection of *RAN1* in P1 and P1A17 using gDNA or cDNA as templates. PCRs were removed from the thermocycler at the cycle number indicated above each lane.

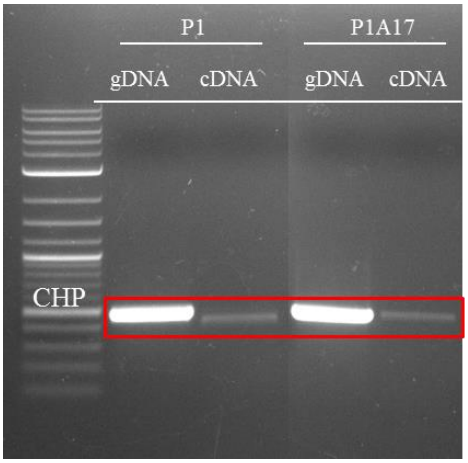


Fig 18. Uniplex PCR detection of *CHP* in P1 and P1A17 using gDNA or cDNA as templates and 30 cycles.

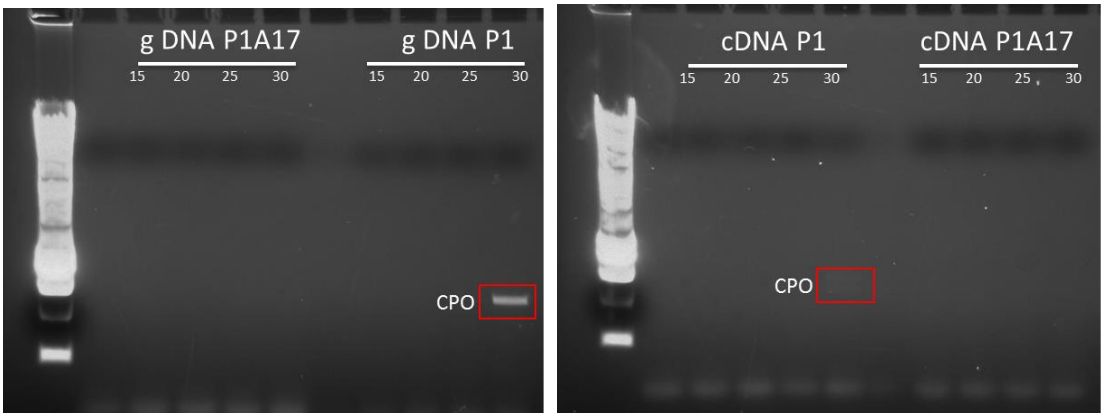


Fig 19. Detection of CPO in P1 and P1A17 with gDNA and cDNA using uniplex PCR. Samples of the PCR reaction mixture were collected at the cycle number indicated above each lane

To determine the role of the *CPO* gene in *P. medicaginis*, knockout (Δ*cpo*) and complementation (*cpo/CPO*) strains were generated by ATMT using pCB_cpo_hph and pCAM-Nat1-CPO vectors, respectively. Seventy candidate Δ*cpo* transformants of strain P1were selected on YPS containing hygromycin (50 mg/ml) media. Among these candidates, only one strain, P1K29, seemed most similar to the nonmelanized pycnidial phenotype of P1A17 (*cpo::TDNA*). Out of eighty-eight candidate complementation

transformants of P1A17, P1A17C23 appeared the most similar to the cultural phenotype of wild type, P1 on SMM.

Gene expression of *RAN1, CPO, CHP,* and *EF1a* was analyzed by multiplex PCR and RT-PCR in knockout and complementation transformants, P1K29 and P1A17C23. In P1K29, the *CPO* gene was not expressed, while *RAN1* and *CHP* were expressed, as in P1A17 (Fig 20). Once again, *CHP* was very weakly expressed in P1K29 as previously shown in P1A17. In complementation strain, P1A17C23, *RAN1*, *CHP* and *CPO* were expressed as in wild type strain P1, though the amplicons of *CHP* and *CPO* were ambiguous in multiplex PCRs, requiring uniplex PCRs of each (Fig 21).

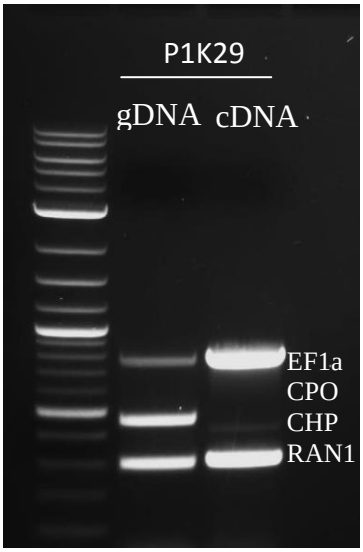


Fig 20. Multiplex PCR and RT-PCR detection of *RAN1, CHP,* and *CPO* in P1K29 (Δ*cpo*) using gDNA and cDNA, respectively. EF1α was used internal PCR control.

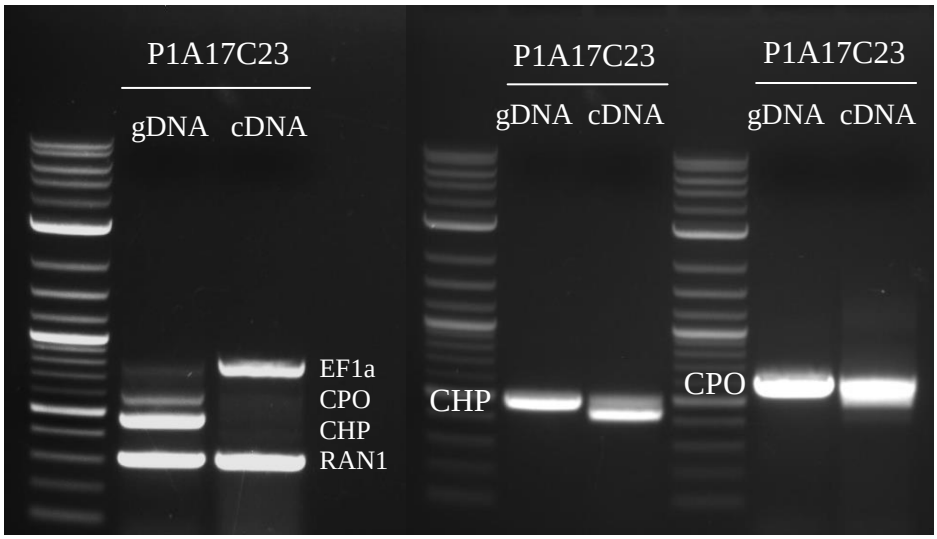


Fig 21. Multiplex PCR and RT-PCR detection of *RAN1*, *CHP*, and *CPO* in complementation strain, P1A17C23 using gDNA and cDNA, respectively (one gel on left). Uniplex PCR and RT-PCR detection of *CHP* and *CPO* in P1A17C23 using gDNA and cDNA, respectively (two gels on right). *EF1α* was used internal PCR control.

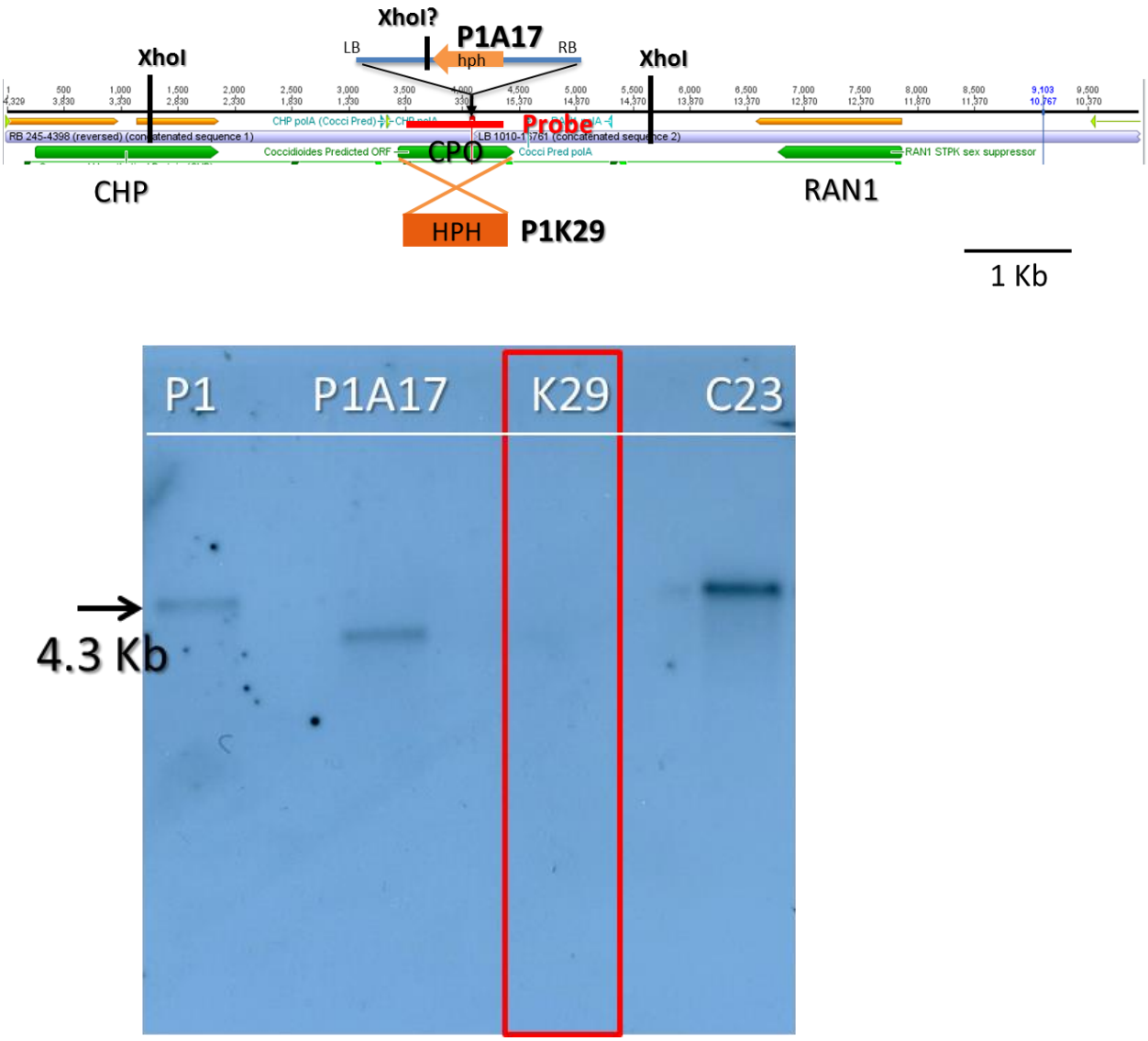


Fig 22. Verification of knockout and complement of *CPO* by Southern blotting A: Schematic representation of *XhoI* restriction enzyme sties and disruption by T-DNA in P1A17 and deletion by pCBCPO-hph in P1K29 sites. B: Southern blot analysis of the wild type and transformants using deleted region of *CPO* probe. Genomic DNA was digested with *XhoI* prior to blotting.

The Knockout and complementation strains were analyzed by Southern hybridization (Fig 22). The deleted region of CPO in knockout strain (P1K29) was used as probe. The targeted deletion of this region was confirmed by the absence of a band, while single

copies of *CPO* were observed in P1, P1A17 and P1A17C23 using probe. The size of the detected *Xho*I fragment was estimated at 4.3 Kb.

High frequency of homologous recombination using complementation vector

The complementation vector pCAM-Nat1-CPO was used to transform P1A17. The resulting complementation strains, P1A17C19 and C21, lost the selectable marker gene for hygromycin resistance, *hph*, from the T-DNA of P1A17, apparently through homologous recombination at the site of the original T-DNA. In exchange, these complementation strains acquired the nouseothricin resistance gene, *Nat1*, based on growth on YPS media supplemented with nourseothricin (Ntc^{+}) (50 ug/ml) (Fig 22).

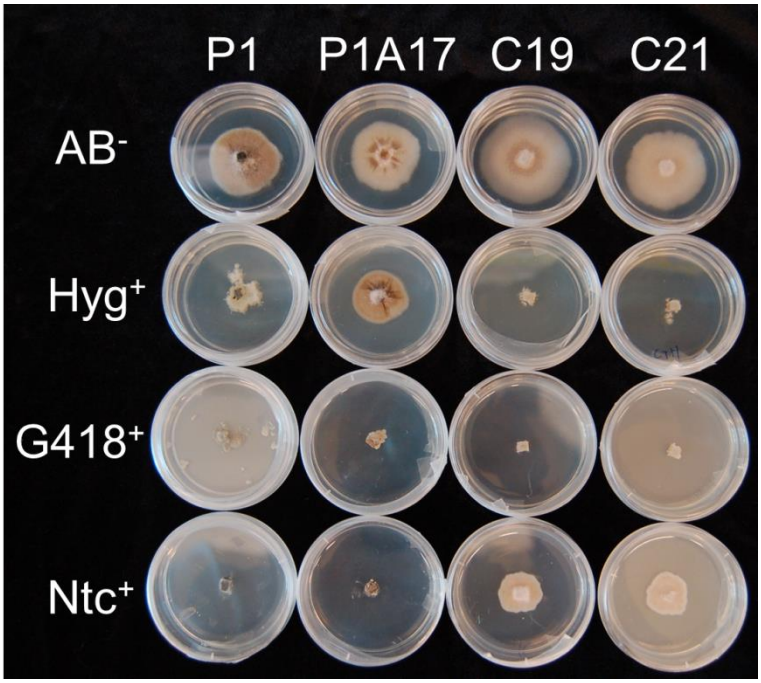


Fig 23. Cultural phenotypes of wild type P1, original TDNA tagged mutant P1A17, and complementation strains, C19 and C21 on YPS alone (AB^{-}) or YPS supplemented with hygromycin B (Hyg^{+}), G418 ($G418^{+}$), or nourseothricin (Ntc^{+}). Hygromycin resistance (Hyg^{R}) was lost from C19 and C21.

This apparent homologous recombination between original T-DNA of P1A17 and the second T-DNA of the complementation vector occurred at high frequency, up to 72% (Table 4). However, this ratio of recombination was not consistent. In control transformations with the 'empty' pCAM-Nat1 vector, the frequency of homologous recombination was similar (79%) to the highest frequency achieved using the complementation vector (Table 4 & 5). However, transformation efficiency (number of transformants produced) using the empty vector was 11-fold higher than the complementation vector carrying wild type genomic DNA (*CPO* gene) (Table 5).

Table 4. Frequency of original TDNA replacement in P1A17 when transformed with complementation vector pCAM-Nat1-CPO, based on loss of hygromycin resistance (Hyg^R). The results of three ATMT experiments are present.

Recipient mutant	Homologous[1]			Ectopic[2]		
	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3
P1A17	8/22, 36%	26/36, 72%	24/44, 55%	14/22, 64%	10/36, 28%	20/44, 45%

[1] Hyg^R lost, [2] Hyg^R and Ntc^Rpresent

Table 5. Frequency of gene replacement using 'empty' vector pCAM-Nat1 based on Ntc^R colony phenotypes. All transformants generated during experiment 2 from Table 4.

Recipient mutant	Homologous[1]	Ectopic[2]
P1A17	301/381, 79%	80/381, 21%

[1] Hyg^R lost, [2] Hyg^R and Ntc^Rpresent

Table 6. PCR confirmation of marker gene presence or absence in wild type P1, TDNA-tagged mutant P1A17, and complementation transformant P1A17C23.

	PCR product	Strains		
		P1	P1A17	P1A17C23
Genotype	*trpC-hph*	Absent	Present	Absent (Lost)
	trpC-nat1	Absent	Absent	Present

The *Agrobacterium* binary vector, pBHt2 was used to generate P1A17, resulting in the integration of a TDNA carrying the hygomycin phosphotransferase gene (*trpC-hph*), used as a genetic marker for selection. The loss of the *trpC-hph* marker and acquisition of the *trpC-nat1* marker were confirmed by PCR in complementation strain P1A17C23 (Table 6).

Discussion

Selection of transformants: Gene deletion and complementation have played important role in analysis of the function of genes in fungi. In yeasts, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, short regions (50-100 bp) of homologous DNA flanking a selectable marker cassette are sufficient for high efficiency gene replacement (Wach et al., 1994; STEEVER et al., 1998). However, gene replacement requires relatively long regions (>1,000 bp) DNA in filamentous fungi for homologous recombination frequencies of 10-30% (Asch and Kinsey, 1990; El-Khoury et al., 2008). Moreover, in wild type *Neurospora crassa*, gene replacement via homologous recombination occurred in 3-5% of transformants (Ninomiya et al., 2004). In the current study, the inferred frequency of homologous recombination in the knockout transformants was 0.0007% ($70/10^5$) based on growth on the YPS media containing hygromycin (50 ug/ml), but not on media containing G418 (100 ug/ml). The *nptII* marker conferring G418 resistance in vector pCB-cpo-hph is outside the homologous DNA and thus should not integrate into the gDNA if a double crossover of the homologous flanks occurs. The transformants are able to grow on media with hygromycin, but not on media with G418, indicating that homologous recombination occurred successfully. Among the

70 candidate knockout transformants, only one strain, P1K29, produced completely non-melanized pycnidia as P1A17 did.

In the case of complementation transformants, the transformation efficiency by pCAM-Nat1-cpo was about 0.0004% ($22/10^5$, $36/10^5$ and $44/10^5$). Among 102 complementation candidates, 44 transformants were able to grow on YPS with nouseothricin (50 ug/ml) and hygromycin (50mg/ml), indicating ectopic integrations. The remaining Ntc^R Hyg^S transformants were inferred to have undergone homologous recombination at the insertion site of the original TDNA was 57% (58/102). Similarly, up to 79% of the transformants exhibited homologous recombination at the site of the original TDNA using empty vector, pCAM-Nat1. In filamentous fungi, site-specific homologous recombination generally occurs at less than 1%, while the frequency of homologous recombination increased to 50 to 100% in mutants deficient non-homologous end joining (NHEJ) in *Neurospora crassa* (Kück and Hoff, 2010). Thus, homologous recombination can occur at high frequency between the original T-DNA and the T-DNA of the subsequent complementation vector. The numerous of genetically modified plants (GMP) containing selectable markers such as antibiotic resistance genes have been tested in the field. The acquiring antibiotic resistance genes by bacterial communities from GMP has been controversial as an undesired effect of GMT (Gebhard and Smalla, 1999). This result suggests that secondary ATMT with an empty vector could be used to delete selectable markers from previous TDNAs in transformants, prior to field testing.

Cultural characteristic of wild type and CPO *mutants and complementation strains*: To confirm the role of the *CPO* gene in pycnidial melanization of *P. medicaginis*, the

following set molecular Koch's postulates for gene function were followed (Falkow, 2004):

1. The gene should be present in all phenotypically wild type strains and absent from mutant strains.
2. Specific inactivation of the gene(s) associated with the phenotype should bring about loss of function in the transformant.
3. Complementation of the mutated gene by the reintroduction of the wild-type gene should restore the function (and wild type phenotype).
4. The gene associated with the phenotype should be expressed in wild type strains and not expressed in mutant strains.

P1A17 (*cpo::TDNA*) was generated by ATMT and produced non-melanized pycnidia (Fig 1). To determine if a melanin biosynthetic gene(s) was disrupt, melanin precursors, 1,8-diaminonaphthalene and L-DOPA were supplemented, resulting in restoring melanization of P1A17 on YPS (Fig 2). Similar chemical complementation was shown in *Gibberella zeae* HK24 (Δ*acs2*), a mutant producing fewer mature perithecia was restored with addition of 0.5% 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol, which restored the maturation rate of perithecia (Lee et al., 2011). Similarly, the melanization of appressoria in *Colletotrichum graminicola* Δ*CgPKS1* (polyketide synthase 1) deletion mutants was restored using melanin precursor 1,3,6,8-tetrahydroxynaphthalene (THN) (Ludwig et al., 2014). However, pycnidial melanization in *P. medicaginis cpo* mutated strains, P1A17 and P1K29, was observed to be restored eventually (after 1-2 weeks more than WT) on YPS and SMM without chemical complementation. Moreover, melanization

of pycnidia in complementation strains (*cpo/CPO*) was displayed more clearly on SMM than on YPS. Thus, mutation of *cpo* appears to delay pycnidial melanization, rather than completely preventing it. The wild type *CPO*, therefore, may be involved in regulating the rate of pycnidial melanization.

Melanization in fungi: The fungal melanin has been associated with aspects of pathogenic aggressiveness and tolerance to environmental stresses (Jacobson, 2000). Melanin-deficient mutants of *Colletotrichum* species and *Magnaporthe grisea* were unable to penetrate into host tissues using non-melanized appressoria and thus became avirulent (Howard and Valent, 1996; Tsuji et al., 2003). The melanized appressoria of *M. grisea* strongly adhere to the host surface and mechanically penetrate into the epidermal cells of rice. The melanin deficient mutants cannot build sufficient turgor pressure in appressoria required for penetration (Howard and Ferrari, 1989). However, in previous pathogenicity assays, non-melanized pycnidial strain, P1A17, showed no significant difference in virulence on *Medicago* compared to the wild type strain (Dhulipala, 2007). Similar results were observed in *Ascochyta rabiei* mutants in which the polyketide synthase (PKS) involved in DHN-fungal melanin had been deleted. These nonmelanized mutants (Δ*ArPKS1*) of *A. rabiei* showed no significant reduction in pathogenicity on chickpea (Akamatsu et al., 2010). In addition, while TDNA disruption of a gene involved in cell wall integrity, *LmIFRD*, in *Leptosphaeria maculans* reduced pathogenicity, silencing the same gene showed no significant difference from wild type in ultimate pathogenicity levels attained, though the silenced strain infected earlier (Van de Wouw et al., 2009). The melanin deficient mutants of other phytopathogenic fungi no reductions in

virulence from wild types under laboratory condition (Guillen et al., 1994; Tanabe et al., 1995; Frederick et al., 1999).

The role of melanin in terms of protection from environmental stresses has been characterized in numerous fungi. M.J. Butler and A.W. Day state that melanin protects fungi against irradiation, enzymatic lysis in natural soils, temperature extremes, heavy metals, oxidizing agents, desiccation, and fungicides (Butler and Day, 1998). Specifically, DHN melanin of *Wangiella dermatitidis* and *Alternaria altermata* has been shown to protect against hypochlorite and hydrogen peroxide (Jacobson et al., 1995). Moreover, melanized cells of *Cryptococcus neoformans* tolerated 1000 times more hydrogen peroxide than mutant albino cells (Jacobson and Tinnell, 1993). The Δ*ArPKS1* strain of *A. rabiei* produced non-melanized pycnidia that were more sensitive to UV exposure compared to the wild type, which produced melanized pycnidia (Akamatsu et al., 2010). However, the *cpo* mutant strain, P1A17, which produces non-melanized pycnidia, showed no significant difference in sensitivity to hydrogen peroxide (1 mM) compared to wild-type strain P1 (Fig 3). Also, P1A17 did not appear to be more sensitive to UV irradiation compared to wild-type P1 (data not shown). To determine if *CPO* plays a role stress protection, further experiments using higher levels of UV and oxidizing agent should be performed.

How does CPO *function in regulating melanization and what does it encode? A regulatory intergenic region or novel exon?*: Small and untranslated RNAs have been reported in all kingdoms of life. These RNA molecules perform numerous functions, including regulating gene expression (Argaman et al., 2001). Recent evidence suggests

that non-coding RNAs transcribed from introns and exons probably play important roles in genetic regulatory networks in eukaryotes (Mattick, 2004). In the human genome, transcription of a non-coding pseudogene regulates the translation of its homologous protein-coding gene (Hirotsune et al., 2003). Such non-protein coding RNA (ncRNA) has been reported to regulate chromosome architecture and protein expression, and may be involved in controlling of transcription and alternative splicing (Mattick, 2003). The presumably intergenic region disrupted by T-DNA in P1A17 was located between RAN1 and a conserved hypothetical protein (CHP). DNA sequencing of the insertion region did not show any previously annotated open reading frames (ORFs). However, one ORF was predicted using a FGENESH algorithm trained with the *Coccidioides immitis* genome (www.softberry.com). This *Coccidioides* predicted ORF (CPO), approximately 2 kb from either of the flanking RNA1 and CHP (Dhulipala, 2007). The T-DNA in P1A17 disrupted the transcription of *CPO,* which is expressed in wild type strains. This ORF has not been previously identified as involved in the melanin pathway in fungi.

There are at least two plausible explanations for the function of this novel region. First, *CPO* encodes a novel protein involved in pycnidial melanization. A second possibility is that part of the disrupted region or the entire *CPO* region may be a novel ncRNA regulator of pycnidial melanization. Such ncRNA has been shown to be responsible for co-supppresion, transgene silencing, and RNA interference, suggesting that such a regulatory system with RNA signals functions in higher eukaryotes (Mattick and Gagen, 2001). In *Saccharomyces cerevisiae,* gene groups with identified upstream and downstream *cis*-regulatory sequences have been identified. Such gene groups are conserved in multiple ascomycete species and their *cis*-elements are involved in various

modes of regulation, including altered transcription factor-binding specificity, incorporation of new gene targets into an existing regulatory regions and coopting of regulatory systems to control a different set of genes (Gasch et al., 2004). Taken together, our data indicates that the disrupted region of P1A17 containing *CPO* mediates melanization of pycnidia in *P. medicaginis*. However, the functional form(s) and cellular and subcellular localization of CPO need to be further characterized.

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